EXPRESSION AND CHARACTERIZATION OF WOUND AND ETHYLENE INDUCED GENES, AND CHANGES IN ENZYME ACTIVITIES AND CELL WALL POLYURONIDES IN RESPONSE TO WOUNDING AND ETHYLENE IN PAPAYA AND WATERMELON FRUITS

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LIST OF ABBREVIATIONS

α-Gal	α-galactosidase
β-Gal	β-galactosidase
CDTA	Trans-1,2-cyclohexylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid
LOX	Lipoxygenase
PLD	Phospholipase D
PLC	Phospholipase C
EIS	Ethanol insoluble solids
ACO	ACC oxidase
PG	Polygalacturonase
PME	Pectinmethylesterase
ACS	ACC synthase
UA	Uronic acid
EXP	Expansin
JA	Jasmonic acid
OGA	Oligogalacturonic acid
PCR	Polymerase chain reaction
DDPCR	Differential display polymerase chain reaction
ABA	Abcissic acid

Abstract of Dissertation Presented to the Graduate School
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EXPRESSION AND CHARACTERIZATION OF WOUND AND ETHYLENE INDUCED GENES, AND CHANGES IN ENZYME ACTIVITIES AND CELL WALL POLYURONIDES IN RESPONSE TO WOUNDING AND ETHYLENE IN PAPAYA AND WATERMELON FRUITS

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Studies were performed to determine changes in firmness, cell-wall polyuronides, gene expression; and the activities of cell-wall and membrane hydrolases, and ethylene biosynthetic enzymes in intact and fresh-cut papaya fruit during storage at 5 °C. Significantly higher polyuronide solubility and depolymerization and firmness decreases were observed in fresh-cut fruit as compared with intact fruit during storage. Activities of polygalacturonase , alpha- and beta-galactosidases, lipoxygenase, phospholipase D, and ACC synthase and ACC oxidase increased in fresh-cut fruit, and remained significantly higher than in intact fruit throughout the storage period. Differential display analysis performed on fruit stored for 12 hours resulted in identification of 98 genes differentially expressed in response to fresh-cut processing. The differentially expressed cDNAs, PC18-1, PC17, PC23, PA17, PC18-2, PC18-3, PG23, PC18-4, PA19-3 and PG17 showed significant homologies to mitogen- and stress-activated protein kinases, the A kinase

anchor protein 2, CAAX prenyl protease, lipoxygenase, membrane channel protein, superoxide dismutase, ACC synthase 2, *Homo sapiens* BAC clone GSI-195F7, calmodulin and beta galactosidases, respectively. The partial cDNA clones PA19-1, PA22, PC18-4 and PA19-2 showed no significant homology to any known sequences in the database. Northern blot analysis showed high levels of the expression for most of the clones in the fresh-cut papaya fruit during storage. Data suggest that changes in cell walls and membranes due to increased hydrolase activity contribute to the rapid deterioration of fresh-cut fruit.

Watermelon fruit exhibits acute symptoms of whole-fruit softening and placental-tissue water-soaking on ethylene exposure. An experiment was conducted to address the firmness, mRNA and activities of cell wall and membrane hydrolases and ethylene biosynthetic enzymes, and solubility and mol mass properties of polyuronides in placental tissue in response to ethylene exposure. Watermelon fruit harvested at the immature and full-ripe stages were exposed to 50 $\mu L\ L^{-1}$ ethylene for 6 days at 20 ^{o}C. Placental tissue firmness from ethylene-treated ripe and immature fruit decreased nearly 80% during 6 days of ethylene exposure with extensive polyuronide solubility and depolymerization. The levels of mRNA and activities of polygalacturonase, lipoxygenase, phospholipases C and D, and ACC synthase and oxidase elevated on ethylene exposure, suggest that cell-wall and membrane catabolism contribute to the development of water-soaking.

CHAPTER I
INTRODUCTION

Fresh-cut processing of fruits and vegetables includes all operations such as washing, sorting, trimming, peeling, slicing, coring, and dicing. Fresh-cut fruits and vegetables are biologically and physiologically active in that their tissues are living and respiring. The objective of fresh-cut processing is to deliver to the consumers a fresh-like product with an extended shelf life, and at the same time to ensure food safety and maintain sound nutritional and sensory quality.

Fresh-cut fruits are among the most fragile and perishable of commodities. While fresh-cut commodities possess the quality and sensory attributes of the intact commodity, they have significantly different in terms of physiological behavior. Refrigerated storage is commonly used to extend quality maintenance and shelf life of fresh-cut produce; however, chill sensitivity of fruits and vegetables of tropical origin requires new approaches to ensuring high quality and food safety maintenance.

The physiological basis of texture loss and deterioration in fresh-cut fruits are unknown, but a number of lines of evidence indicate a role for enzymes targeting the cell wall and/or membranes. The senescence-delaying influence of Ca^{+2} and the increased juice leakage during storage of fresh-cut commodities suggest a role for cell walls and membranes in the deterioration of fresh-cut produce and indicate that physiological deterioration of fresh-cut produce possibly results from membrane and/or cell-wall damage. Relatively few studies have addressed the biochemical and molecular events contributing to deterioration of fresh-cut produce, especially in tropical fruits.

Identification of genes and gene products involved in the deterioration of fresh-cut papaya fruit will have significance for extending shelf life of tropical fruits in general; and will provide information of value for the long-term goals of designing or selecting cultivars through genetic transformation with resistance or tolerance to the physiology of deterioration. One objective of our study was to monitor changes in enzyme activity and gene expression patterns in fresh-cut and intact papaya fruit.

Fresh-cut processing (wounding) also induces the production of ethylene, which in turn can lead to responses such as water soaking, accelerated senescence, or abscission of infected organs, likely brought about through induction and expression of wound-related genes. Another objective of this study was to isolate and identify genes up-regulated by exogenously applied ethylene in watermelon fruit, a very ethylene-sensitive system.

The objectives of this research were addressed using a combination of biochemical and molecular approaches, and included an assessment of the activities of cell wall and membrane-targeted hydrolases and of the expression of select genes in response to fresh cutting (papaya) and in response to exogenous ethylene (watermelon).

CHAPTER 2
LITERATURE REVIEW

Introduction

Fresh-cut fruits and vegetables are highly perishable and show rapid tissue deterioration during storage after processing. Fresh-cut tropical fruits including banana (Abe and Watada 1991), and papaya exhibited rapid textural loss during only 2 days storage at 4 °C (O'Connor-Shaw et al. 1994). The senescence delaying influence of Ca^{+2} on shredded carrots (Picchioni et al. 1996) and the increased juice leakage during storage of fresh-cut commodities (Cartaxo et al. 1997) indicate that physiological deterioration of fresh-cut produce possibly results from membrane and/or cell wall damage. In fresh-cut vegetables, enhanced activities of chlorophyll-degrading enzymes in cole slaw (Heaton et al. 1996) and pyrophosphatase-phosphofructokinase in sliced carrots (Kato-Noguchi and Watada 1996) have been noted. A wounding-induced increase in phenylalanine ammonia lyase was associated with browning of the cut surfaces of fresh-cut lettuce (Lopez-Galvez et al. 1996) and carrot roots (Leja et al. 1997). Moreover, wounding triggers an increase in the endogenous levels of the plant growth regulator jasmonic acid (JA) (Creelman et al. 1992, Albrecht et al. 1993, Laudert et al. 1996), and this increase is required for gene activation upon wounding (Pena-Cortes et al. 1993). Application of exogenous JA or its methyl ester at physiological concentrations can induce the expression of a variety of genes (Mason and Mullet 1990, Farmer et al. 1992). In potato and tomato, protease inhibitor genes can be activated by oligosaccharide fragments generated from both plant and pathogen cell walls by the action of polygalacturonases (Bishop et al. 1981) and by

the 18-amino acid polypeptide systemin (Pearce et al. 1991). Wounding also induces the production of ethylene, which in turn can lead to responses such as water-soaking, accelerated senescence, or abscission of infected organs, likely brought about through induction and expression of wound-related genes (Abeles et al. 1992). Wound-induced ethylene production in fruit and vegetable tissues has been extensively reviewed (Rosen and Kader 1989, Paull et al. 1997). An increase in ethylene production in response to fresh-cut processing has been observed in papaya fruit within 24 hours (Paull et al. 1997). Rosen and Kader (1989) found an increase in ethylene production of sliced strawberry. The requirement of ethylene in mediating wound-induced gene activation has been demonstrated in tomato (O' Donnell et al. 1996), in which ethylene acts with jasmonic acid to regulate *Pin* 2 gene expression.

Shimokawa (1973) and Risse and Hatton (1982) demonstrated that exposure of watermelon fruit to ethylene at concentrations as low as 1 μL/L brought about placental tissue deterioration and rendered the fruit unfit for consumption. Ethylene-treated fruit were soft, water-soaked, and had off-odors. Ethylene treatment also had an adverse effect on pre-ripe melons. Acute symptoms of softening and water-soaking in placental tissue of ethylene-treated watermelon fruit were also observed by Elkasif and Huber (1989).

Studies have shown that the softening of a number of fruit types during ripening is the result of enzymic hydrolysis of cell wall polysaccharides (Huber et al. 2001, Wakabayashi 2000). Ethylene plays a major role in enhancing the activity of enzymes responsible for changes occurring in cell walls during ripening. Sawamura et al. (1978) reported that exposure of tomato fruit to ethylene advanced the appearance of "pectinase" activity. Ethylene hastened the appearance of C_x-cellulase activity in some fruit types

(Pesis et al. 1978, Awad and Yang 1979). The appearance of C_x-cellulase in avocado fruit (Tucker and Laties 1984) and PG in tomato fruit (Biggs et al. 1986, Grierson et al. 1986) may be transcriptionally regulated. An increase in PG activity was also observed in watermelon fruit in response to ethylene exposure (Elkashif and Huber 1988a). Exposing of various plant tissues to ethylene also causes an increase in membrane permeability (Ferguson et al. 1980, Hanson and Kende 1975, Suttle and Kende 1980, Elkashif and Huber 1988b).

Papaya fruit are climacteric; they undergo a pronounced increase in respiration coincident with ripening (Selvaraj et al. 1982, Sankat and Maharaj 1996). An increase in ethylene production parallels the respiration rise, attaining a maximum simultaneously with the respiratory climacteric (Paull and Chen 1983, Lazan et al. 1990, Ali et al. 1999). Studies conducted on ethylene production in papaya have included measurement of key enzymes, 1-aminocyclopropane 1-carboxylate (ACC) synthase (EC 4.4.1.14) and ACC oxidase. Activities of ACS and ACO increase during papaya fruit ripening (Chan et al. 1990, Ali et al. 1999) and both appear to be highly regulated at the transcriptional level (Mason and Botella 1997, Lin et al. 1997, Zainal et al. 1999, Ali et al. 2000). In the cv. Eksotica papaya, *pACO 1* and *carERS 1* genes, encoding for an ACC oxidase and ethylene receptor, respectively, have been isolated and characterized (Ali et al. 2000). Expression of these genes was upregulated during ripening, before the expression of ripening-specific cell-wall genes including β-galactosidase (EC 3.2.1.23) *PBG2* and xyloglucan endo-transglycosylase (EC 2.4.1.207) *carXET1* genes (Lazan et al., 2000).

Ethylene Biosynthesis

Ethylene is the plant hormone that regulates fruit ripening by coordinating the expression of genes. The sharp increase in climacteric ethylene production at the onset of

ripening is believed to be the initiator of changes in color, texture, aroma and flavor; and changes in other biochemical and physiological attributes (Brady 1987, Lelievre et al. 1997). The ethylene biosynthetic pathway is established as methionine → SAM → ACC → ethylene (Adams and Yang 1979). The conversion of SAM to ACC is catalyzed by ACC synthase, while the subsequent oxidation of ACC to ethylene is catalyzed by ACC oxidase. Both ACC synthase and oxidase are limiting in preclimacteric fruit but are greatly induced during ripening (Lelievre et al. 1997).

Cloning and partial characterization of members of the ACO and ACS gene families show that gene family members are differentially expressed, both spatially and temporally. For example, among three ACO gene isoforms detected in tomato (Bozayen et al. 1993), *LE-ACO1* and *LE-ACO3* are expressed during early stages of tomato fruit ripening, and *only LE-ACO1* continues to be expressed during ripening. The ACO proteins encoded by these genes are highly homologous, while ACO genes share identical numbers and positions of introns. The ACO cDNA clones were also isolated from many other fruits including melon *(cucumis melo)* (Balague et al. 1993) peach (Callahan et al. 1992) and apple (Dong et al. 1992).

ACS is encoded by a highly divergent multigene family (Rottmann et al. 1991), and differentially expressed in response to various signals. Nine ACS genes detected in tomato (Zarembinski and Theologies 1994) were expressed differentially during ripening and in response to various signals including wounding (reviewed in Lelievre et al. 1997 and Jiang and Fu 2000). Among these, the most abundant mRNA species corresponds to the *LE-ACS2* gene and to a lesser extent *LE-ACS4* (Lincoln et al. 1993, Olson et al. 1991, Yip et al. 1992). The cDNAs of *LE-ACS2* and *LE-ACS4* are greatly induced before and

during climacteric fruit ripening (Yip et al. 1992, Lincoln et al. 1993). Wound-inducible genes for ACC synthases have been cloned from many plants including tomato leaves (Shiu et al. 1998), Arabidopsis leaves (Liang et al. 1992), and melon fruit (Yamamoto et al. 1995).

Ethylene perception begins with the binding of the gaseous phytohormone to a receptor, a membrane-associated protein and homolog to the Arabidopsis thaliana ETR 1 (ethylene response 1) protein (Blecker and Schaller 1996). The ETR1 gene encodes a protein showing homology to the bacterial histidine kinase proteins involved in the two-component system of signal transduction in bacteria. The functional ETR1 protein is a disulfide-linked dimer with an N-terminal sensor domain adjacent to the histidine kinase domain, with a putative receiver domain in the C-terminal region (Chang 1996). The binding of ethylene to the sensor domain of ETR 1 on the external site of the plasma membrane would result in conformational changes to the histidine kinase protein domain, located on the cytoplasmic site of the membrane, and transduces a cascade of downstream responses (Blecker and Schaller 1996, Chang and Shockey 1999, Hall et al. 2001). A tomato homolog of ETR1, NR, has been cloned from the tomato-ripening mutant *Never ripe* (Wilkinson et al. 1995). This mutant is defective in ethylene perception and does not accumulate NR mRNA (Payton et al. 1996). *Never ripe* exhibits low ethylene production compared with wild type fruit (Lanahan et al. 1994). Based on studies of *Never ripe* mutants, it has been suggested that the number of ethylene binding sites increases during ripening and that NR transcript accumulation is both developmentally and ethylene regulated (reviewed in Lelievre et al. 1997). Heterologous expression of the mutant Arabidopsis ETR1 gene in tomato was shown to cause

significant delays in fruit ripening and flower senescence (Wilkinson et al. 1997), indicating that the pathway of ethylene recognition and response is highly conserved. The CTR1 gene product acts downstream of ETR1 and seems to be a negative regulator, as a loss of gene function results in a constitutive ethylene response (Kieber et al. 1993). The CTR1 gene encodes a putative serine/threonine kinase related to the MAPKK kinase family (Kieber et al. 1993). A CTR1 homolog was isolated from tomato fruit that is upregulated during ripening and in response to ethylene treatment (Giovannoni et al. 1997). In all of the transgenic plants obtained with reduced ethylene synthesis, the ripening phenotype is altered and can be at least partially reversed by continuous exposure to exogenous ethylene or propylene (reviewed in Lelievre et al. 1997). In ACO antisense melons (*Cucumis melo*), with only 0.5% of wild-type ethylene production rates, however, fruit softening was completely suppressed (Guis et al. 1997). Several ethylene response genes including E4, E8, J49 have been characterized from tomato fruit (reviewed in Lelievre 1997). Ethylene has also been reported to regulate the expression of polygalacturonase (EC 3.2.1.15) and endo-β-1-4-glucanase genes (*Cel1* and *Cel2*) (Lelievre 1997).

Cell-Wall Modifications during Fruit Ripening

Fruit cell walls undergo significant ultrastructural and biochemical changes during ripening. Loss in density of primary cell wall, leading to cell separation, has been reported in many fruits including apple (Arie et al. 1979), tomato (Hobson 1981, Crookes and Grierson 1983), pear (Arie et al. 1979), strawberry (Knee et al. 1977) and plum (Redgewell et al. 1997). While all of the major cell wall polysaccharides undergo modifications during ripening, the amplitude of the changes differs among fruit types. Increased solubility, depolymerization, de-esterification and loss of neutral sugar side

chains are the most common phenomena observed. For example, increased pectin solubility during ripening has been reported in many fruits, including mango (Mitcham and McDonald 1992), tomato (Gross and Wallner 1979), peach (Karakurt et al. 2000), persimmon (Cutillas-Iturralde et al. 1993), pepino (O'Donoghue et al. 1997), cherry (Batissee et al. 1994), nectarines (Lurie et al. 1994), strawberry (Redgewell et al. 1997) and papaya (Lazan et al. 1995). The magnitude of solubility increases varies greatly among different fruits. Water-soluble polyuronides range from 10% of total cell-wall uronic acid content in ripe grapefruit (Hwang et al. 1990) to 85% in ripe avocados (Wakabayashi et al. 2000). Depolymerization and degalactosidation are believed to be the primary contributors to increased pectin solubility in most fruits (Huber et al. 2001). Carrington et al. (1993) have reported a significant decrease in water-soluble pectins in transgenic tomato fruit with reduced levels of polygalacturonase. Redgewell et al. (1992) reported, however, that depolymerization and degalactosidation were not required for the initial solubilization of pectins in ripening kiwifruit. Pectin solubility can also be significantly influenced by cell-wall isolation method (Huber 1990).

Pectins have been classified on the basis of methods used to remove them from insoluble cell wall isolates. The so-called water-soluble pectic fraction includes polymers that are freely soluble in water or weak buffer extracts of isolated cell walls. Chelator-soluble pectins are polymers held in the wall by ionic interactions. The Na_2CO_3-soluble fraction, extracted after the removal of water- and chelator-soluble pectins, consists of covalently-bound pectins presumably removed by hydrolysis of weak ester linkages. Subsequent extractions with increasingly strong alkali releases polysaccharides through hydrolysis of ester linkages, by disrupting inter- and intramolecular hydrogen bonds

between hemicelluloses and cellulose microfibrils. The 1 M KOH-soluble fraction contains significant amount of pectic in addition to hemicellulosic polysaccharides; whereas later extraction with 4 M KOH releases polymers highly enriched in hemicelluloses (e.g. glucomannans and xyloglucans) (Coimbra et al. 1996, Rose et al. 1998).

In addition to changes affecting pectic polymers during ripening, a loss of neutral sugars from the cell wall, primarily galactose and arabinose, has been reported for many fruits (Knee et al. 1977, Gross and Wallner 1979, Ahmed and Labavitch 1980, Seymour et al. 1990, Dawson et al. 1992, Brett and Waldron 1996, Rose et al. 1998, Chin et al. 1999). A temporal association between pectin solubilization and degalactosidation has been observed in the early stages of melon fruit ripening (Rose et al. 1980). In the late stages of tomato ripening, there is evidence that xylosyl and glucosyl residues continue to be incorporated into the cell wall, suggesting that sustained or renewed polymer synthesis might contribute to ripening-specific changes in cell walls (Tong and Gross 1988, Mitcham et al. 1989, Greve and Labavitch 1991, Seymour and Gross 1996).

Hemicellulosic modifications, particularly depolymerisation and/or loss of hemicelluloses, have been reported during ripening of many fruits, including tomato (Cheng and Huber 1997), avocado cv. Haas (Sakurai and Nevins 1997), mango (Mitcham and McDonald 1992), papaya (Lazan et al. 1995), Charentais melon (Rose et al. 1998) and persimmon (Cuttillas-Iturralde et al. 1994), and were attributed to changes in xyloglucans, the predominant hemicellulosic polysaccharides in dicotyledonous plants. Xyloglucan degradation is often accompanied by extensive breakdown of polyuronides in ripening fruit, possibly due to increased hydrolase mobility (Wakabayashi 2000).

However, there were no or little changes in the molecular weight profiles of xyloglucans during ripening of apple cv. Braeburn (Percy et al. 1997), tomato var. 83-G-38 (Maclachlan and Brady 1994) and avocado cv. Lula (O'Donoghue et al. 1997). Other hemicellulosic polymers including xylan, arabinoxylan, mannan and galactoglucomannan are generally minor components in most fruits and the changes in these polysaccharides during ripening are unknown (Rose et al. 1998).

The distribution of organic acids and inorganic ions and apoplastic pH also undergo significant changes during fruit ripening. The apoplastic pH of pre-ripe fruits is approximately neutral and declines as much as three units during ripening of tomato (Almeida and Huber 1999), peach and apricot (Ugalde et al. 1988). Cell-wall acidification could result from de- esterification or through the action of proton pumps such as galactose-$^{+}$H symporter in *Chlorella* cells (Staddler et al. 1995, Seymour and Gross 1996). An increase in ATPase activity has been observed during ripening of strawberry (Lurie and Ben-Arie 1983), apple (Ben-Arie and Faust 1980) and pepino (Heyes et al. 1997). Loss in compartmentation between symplast and apoplast possibly due to lipid catabolism and oxidative stress has been noted during ripening of grape berries (Lang and During 1991) and strawberries (Pomper and Breen 1995). Brady (1987) proposed that leakage of organic acids into the cell wall might remove pectin-bound calcium, but MacDougall et al. (1995) later determined that the levels of organic acids found in the wall were insufficient to disrupt such crosslinkings. The levels of soluble calcium detected in apoplast fluid isolated from tomato (Ruan et al. 1996), including throughout ripening (Almeida and Huber 1999), are more than adequate to suppress the activity of PG (Rushing and Huber 1990). Apoplastic pH influences

turgidity and wall loosening possibly through inhibition of hydrolytic reactions and intermolecular interactions between structural carbohydrates and proteins (Rayle and Cleland 1992). The apoplastic environment also affects reactions involved in pathogenesis and elicitor metabolism (De Lorenzo et al. 1991, Knogge 1996). Heyes et al. (1997) and Huber and O'Donoghue (1993) have proposed a role of apoplastic pH in influencing fruit textural changes possibly by modifying the catalytic behavior or activity of cell wall hydrolases. In vitro studies have demonstrated that catalytic behavior and binding of several cell wall enzymes are modified in response to alterations in pH, ionic composition and ionic strength (Ricard and Noat 1986, Nari et al. 1986). The modification of cell-wall hydrolase activities by alterations in pH and ionic composition has also been reported by Almeida and Huber (1999), who suggested that pH changes could serve as a means of temporally regulating the sequence of action of specific proteins during tomato fruit ripening. Ionic strength and composition can affect protein binding and kinetics as a result of shielding effects on the protein charges and on polyelectrolytic substrates (Nari et al.1986, Crasnier et al. 1985).

Reactive oxygen species (ROS) have been shown to be involved in polysaccharide depolymerization. The hydroxyl radical has been reported to cause the scission of polysaccharides such as hyaluronate (Hawkins and Davies 1996), chitosan (Tanioka et al. 1996), and pullulan (Crescenzi et al. 1997). Miller (1986) reported a decrease in viscosity and generation of reducing sugars in response to incubation of cell wall polymers in 1 mM H_2O_2 at pH 6.5. Extensive hydrolysis of pectin, xyloglucan and other polysaccharides from tomato and cucumber fruits was observed when cell polymers were incubated with ascorbate, Cu^{2+}, and H_2O_2, possibly due to hydroxyl radical

formation in a Fenton-type reaction (Fry 1998). Furthermore, the author has demonstrated that polysaccharides are susceptible to nonenzymic scission under physiologically relevant conditions. The availability of Cu^{+2}, ascorbate and H_2O_2 in plant apoplastic fluid, provides conditions suitable for hydroxyl radical generation which have been proposed to participate as site specific oxidants in cell wall loosening, during fruit ripening and organ abscission. Fry (2000) also hypothesized that ROS-mediated scission of polysaccharides-may lead to the production of oligosaccarides with structures unrelated to fragments generated by hydrolytic enzymes. The uniqueness of fragments generated via either mechanism (hydrolytic vs lytic) might have implications regarding the eliciting or growth regulator potential, and gene induction capacity of these oligosaccharides.

Cell-Wall Enzymes

Polygalacturonase (PG)

Three PG isoforms designated as PG1, PG2a and PG2b have been reported from tomato fruit (Ali and Brady 1982), and are derived from a single mRNA, which undergoes post-translational processing and glycosylation of the PG polypeptide, or by interaction with other proteins (Smith et al. 1988, 1990). The PG2 isoforms possess the same catalytic polypeptide, glycosylated to slightly different extents (Ali and Brady 1982). On the other hand, PG1 was found to be composed of one unit of PG2 tightly bound to a non-catalytic aromatic amino acid rich glycoprotein, termed the β-subunit (Pogson et al. 1991). PG1 was detected during early stages of tomato fruit ripening while the other two isoforms were found to increase until they became the dominant forms in ripe tissues (Brady et al. 1983). Two PG isoforms of 46 and 48 kDa were also isolated from ripe avocado fruit (Wakabayashi and Huber 2001). However, there was no evidence

with avocado fruit of a PG isoform resembling tomato fruit PG 1, a heterodimer of tomato PG2 and the aromatic amino acid-rich glycoprotein, the β-subunit (DellaPenna et al. 1996). Transgenic plants with reduced levels of the β-subunits demonstrated enhanced pectin solubilility and depolymerization, indicating that the β-subunit may play a role in limiting PG action in vivo (Zheng et al. 1992, Watson et al. 1994). Evidence for a role of the β-subunit in tomato fruit softening and in controlling the mobility of PG2 in vivo was also reported by Chun and Huber (2000).

PG is classified as endo-PG (EC 3.2.1.15) or exo-PG (EC 3.2.1.67) based on substrate specificity and mode of action. Endo-PG catalyses internal hydrolytic cleavage of the unesterified α-1,4-D-galacturonan linkages, whereas exo-PG releases terminal galacturonosyl residues from the non-reducing end of pectin. Endo-PG is the most thoroughly characterized among the wall hydrolases, and has been detected in many fruits, such as tomato (Ali and Brady 1982), peach (Lester et al. 1994), cucumber (Saltveit and McFeeters 1980), avocado (Awad and Young 1979, Wakabayashi et al. 2000) and apple (Knee and Bartley 1981). In some fruits such as papaya, guava, carambola, and mango (Chin et al. 1993), however, only exo-PG activity was detected. Correlation between PG activity and pectin degradation has been shown during ripening of many fruit including tomato (Ali and Brady 1982), pear (Ahmed and Labavitch 1980), avocado (Wakabayashi et al. 2000), papaya (Lazan et al. 1990), mango (Ali et al. 1991) and peach (Downs et al. 1992).

In tomato fruit expressing an antisense PG gene with PG levels of 1% compared to wild type, pectin solubility remained at wild type levels whereas pectin depolymerisation was reduced (Smith et al. 1990). The transgenic fruit did not exhibit

abnormal ripening behavior, indicating that PG-dependent pectin degradation is neither required nor sufficient for tomato fruit softening (Smith et al. 1988, Giovannoni et al. 1989). These fruit also exhibited enhanced fruit quality, such as resistance to mechanical damage and cracking (Schuch 1991, Kramer et al. 1992), particularly during over-ripening. The first transgenic tomato fruit containing antisense down-regulated levels of PG was commercialized briefly as FLAVR SAVR (Kramer and Redenbaugh 1994) and has found use in the tomato processing industry (Schuch 1994). Expression of PG in the non-softening tomato mutant, rin, also failed to induce softening despite the occurrence of normal levels of pectin depolymerization and solubilility increases (Giovannoni et al. 1989).

Pectin methylesterase (PME)

PMEs remove methyl groups from the C-6 position of esterified galacturonic acid polymers. These enzymes can act synergistically with PG, generating sites for PG action (Wegrzyn and MacRae 1992, Giovanne et al. 1994); however, the extent of such interactions in situ during ripening is unclear (Tucker 1993). Two major groups of PME have been identified in tomato based on their isoelectric points and location in tomato plant: Group 1 with isoelectric points (pls) of 8.2, 8.4, and 8.5 are restricted to fruit tissues whereas group 2 isoforms with pl values of 9 and above are found in both fruit and vegetative tissues (Gaffe et al. 1994).

PME activity has been detected in numerous fruits, but its correlation with tissue softening is not clear. The trends in PME activity during ripening depend greatly on fruit type. PME activity increased during ripening of tomato (Tucker et al. 1982), papaya (Paull and Chen 1983), guava (Lazan and Ali 1993), carambola (Chin 1993, 2000, Chin et al. 1999), and goldenberry (Trincherro et al. 1999), but has been reported to decrease

in avocado (Awad and Young 1979), mango (Roe and Bruemmer 1981, Lazan and Ali 1993) and bell pepper (Sethu et al. 1996). PME activity was found to remain constant during ripening of pear (Ahmed and Labavitch 1980). Such variations may be due to the presence of multiple isoforms (Tucker et al. 1982) and/or endogenous enzyme inhibitors (Baletrieri et al. 1990). Tomato plants transformed with an antisense PME gene showed nearly complete inhibition of PME activity, but fruits softened with reduced pectin solubility, esterification and depolymerization (Tieman et al. 1992, Hall et al. 1993). The loss of tissue integrity observed in these fruits was attributed to the higher esterification levels of pectins, reducing the number of potential calcium-binding sites (Tieman and Handa 1994).

Galactosidases

Loss of galactosyl residues from the cell wall is one of the most universal events during ripening of many fruits, such as tomato, melon, squash, muskmelon, watermelon, strawberry, kiwifruit and persimmon (Redgewell et al. 1997, Cutillas-Iturralde 1993, Seymour and Gross 1996). β-galactosidase activity has been shown to increase during ripening of many fruits including apple (Bartley 1974), tomato (Watkins et al. 1988), papaya (Lazan and Ali 1993), kiwifruit (Wegrzyn and MacRae 1992), and mango (Ali et al. 1990, Lazan and Ali 1993). Beta-galactosidases are typically assayed using p-nitrophenyl β-D-galactopyranoside, against which they show very high activity. In contrast, they show little activity towards isolated cell wall polysaccharides (Huber et al. 2001). The cell wall-active isoforms have been reported to represent exo-β-D-galactanases rather than true β-galactosidases since enzymes of the latter group are relatively inactive toward polymeric substrates (Huber et al. 2001). Some β-galactosidases are active toward hemicelluloses (Ranwala et al. 1992, Li et al. 2001),

releasing monomeric galactose (Li et al. 2001). β-galactosidases purified from muskmelon (Ranwala et al. 1992), avocado (De Veau et al. 1993), kiwifruit (Ross et al. 1993), Japanese pear (Kitagawa et al. 1995), tomato (Carey et al. 1995; Carrington and Pressey 1996), papaya (Ali et al. 1998) and carambola (Chin et al. 1999) fruits have been reported to degrade isolated polymers derived from cell wall, probably by cleaving β-1-4 galactan bonds. The purified enzyme from kiwifruit released galactose from a carbonate-soluble fraction and a galactan (Ross et al. 1993), of which both polymers contain 1,4-linked galactosyl residues (Redgewell et al. 1992). The enzyme was also able to release small amounts of galactose from xyloglucan and galactoglucomannan (Ross et al. 1993). Although the effect of degalactosidation on the physical properties of pectins is not understood, mol mass downshifts in isolated pectin fractions have been reported for avocado (de Veau et al. 1993) and muskmelon (Ranwala et al. 1992) β-galactosidases.

β-galactosidase is available in multiple forms in fruit tissues but all isoforms can not degrade native cell wall substrates. Smith and Gross (2000) have detected at least seven β-galactosidase genes in tomato, 6 of which were reported to deglycosylate tomato fruit cell-wall fractions enriched in β-(1,4)-D-galactans. All the β-galactosidase isoforms purified from Japanese pear (Kitagawa et al. 1995) were active as assayed by galactose release at varying degrees against native cell wall polysaccharides. One of these enzymes, β-galactosidase 1, was able to act on all fractionated polymers of native cell wall (CDTA-, Na_2CO_3-, guanidine thiocyanate- and KOH-soluble fractions) of Japanese pear and arabinogalactan from larch wood, whereas β-galactosiadse 2 was active against KOH-soluble polymers and arabinogalactan. Similar observations were noted with β-galactosidase from papaya (Ali et al. 1998). β-galactosidase isoforms from papaya fruit

showed differential abilities to modify cell wall from unripe papaya fruit. Whereas β-galactosidase 1 and 3 showed significant activity towards hemicelluloses, β-galactosidase 2 preferentially degraded pectic polymers (Ali et al. 1998). All three isoforms of papaya β-galactosidase exhibited exo-galactanase activity. β-galactosidase has also been proposed to contribute to membrane galactolipid modification through hydrolytic as well as transfer reactions (Onishi and Tanaka 1995, Yoon and Ajisaka 1996, Kajihara et al. 2000, Zeng et al. 2000).

Plant α-galactosidase shows both acidic and alkaline pH optima and often exists in multiple forms. Much of the earlier studies have focused on storage organs, such as seeds, where the enzyme may induce seed germination in legumes (Keller and Pharr 1996) and deficiency of the enzyme is thought to inhibit germination in coconut (Mujer et al. 1984). The role of α-galactosidase in storage organs has been reported to be the mobilization of α-D-galactosyl-containing oligo and polysaccharides, particularly the raffinose oligosaccharides (Keller and Pharr 1996, Castonguay and Nadeau 1998) and galactomannan (Brett and Waldron 1996). Another possible role for α-galactosidases is to protect plants from phytotoxic substances produced by invading microorganisms (Dey and del Campillo 1984). α-galactosidases are also implicated in the metabolism of galactolipids where the enzyme assists galactolipid degradation and membrane disintegration during cell wall lysis, following injury, or during senescence (Sastry and Kates 1964, Dey and delCampillo 1984).

The role of α-galactosidase in fruits is largely unknown. It has been detected in many fruits including tomato (Wallner and Walker 1975), Bartlett pear (Ahmed and Labavitch 1980), watermelon (Itoh et al. 1986), apple (Dick et al. 1990), avocado (De

Veau et al. 1993), grapefruit (Burns and Baldwin 1994), papaya (Lazan et al. 1995), and carambola (Chin et al. 1999). Relatively few purification studies have been attempted. Purified α-galactosidases from watermelon (Itoh et al. 1986) and sweet melon (*Cucumis melo*) fruits (Gao et al. 1999) have acidic pH optima, while the optimum pH of the enzyme from coconut endosperm (Mujer et al. 1984), and two other isoforms from sweet melon fruit (Gao et al. 1999) were alkaline. α-galactosidase purified from avocado fruit has been shown to degrade tomato fruit pectin in vitro (De Veau et al. 1993). Itoh et al. (1986) has reported that watermelon α-galactosidase is capable of hydrolysing glycoprotein and glycolipids. α-galactosidase activity correlated closely with the softening process during ripening of papaya fruit (Ali et al. 1998). In olive fruit, α-galactosidase activity was not detected until the fruit were fully ripe (Heredia et al. 1993). Moreover, a temporal correlation between α-galactosidase activity and the decrease in non-cellulosic cell wall neutral sugars during ripening of olive fruit was noted, and the treatment of the wall with high salt and then measurement of the residual activity suggested that the enzyme was wall-associated (Heredia et al. 1993, Fernandez-Bolanos et al. 1995).

Expansins (EXP)

Expansins are newly discovered wall-modifying proteins. These proteins do not appear to possess hydrolytic activity but are able to induce wall loosening through interaction with cellulose (Cosgrove 2000a). They apparently function to disrupt hydrogen bonding (McQueen-Mason et al. 1992, McQueen and Cosgrove 1994, Cosgrove 2000a, 2000b). Expansins have been proposed to be responsible for acid-induced wall extension in oat coleoptiles (Cosgrove and Li 1993, Li et al. 1993). They act catalytically to stimulate wall expansion in vitro (Cosgrove 2000a) and in living cells

when applied exogenously to Arabidopsis thaliana hypocotyls and cucumber root hairs (Moore et al. 1995), tomato meristems (Fleming et al. 1999), and tobacco cell cultures (Link and Cosgrove 1998).

Expansins are categorized into two families, α and β expansins, based on substrate preferences (Cosgrove 2000a, 2000b). The α-expansins were more active on a range of dicot and monocot cell walls, but less effective on cell walls from grasses (McQueen-Mason et al. 1992). However, β-expansin is more active on grass cell walls (Cosgrove et al. 1997). Both expansin families consist of a series of conserved cysteines in the N-terminal region of the protein, a HFD (histidine, phenylalanine, aspartate) motif and an FRRV (phenyalanine, arginine, arginine, valine) motif in the middle of the protein, and a series of tryptophans (W) near the carboxy-terminus (Cosgrove 1997). α- and β-expansins are large multigene families (Cosgrove 2000a). α-expansins from diverse taxonomic groups including a gymnosperm (Hutchinson et al. 1999) and a fern (Kim et al. 1999) show high sequence similarity, indicating that the biochemical function of these proteins is highly conserved (Cosgrove 2000a). An α-expansin gene, *Le-EXP1*, was reported to be expressed specifically in the later stages of ripening in tomato fruit and its expression was stimulated by ethylene (Rose et al. 1997). In transgenic tomato fruit with an antisense *Le-EXP1* construct, softening was reduced while over-expression of gene resulted in increased softening during ripening (Brummell et al. 1999, Cosgrove 2000b) indicating a contribution of the protein in tomato fruit softening. Fruit under-expressing *Le-Exp1* protein exhibited suppressed pectin depolymerisation during late ripening, but showed normal downshifts in hemicellulose mol mass. Fruit over-expressing *Le-Exp1* were softer when pre-ripe and during ripening, exhibited unaltered pectin metabolism,

but enhanced breakdown of hemicelluloses. In one contrasting report, Hayama et al. (2000) observed no differences in transcript abundance or immunologically detected levels of a ripening-specific expansin between melting-flesh and stony-hard peach cultivars. Expansin-like activity has also been reported in pepper, avocado and pear (Rose et al. 2000), and strawberry (Civello et al. 1999) fruits.

Wound Metabolism

Wounding of plant tissue stimulates biochemical cascades directed at healing and furthering defense responses. Responses to mechanical damage can be local, systemic, or both and hence involve the generation, perception, translocation, and transduction of wound signals to activate the expression of wound-inducible genes. A central role for jasmonic acid (JA) in plant responses is well known (Farmer and Ryan 1990); however, many other compounds, including the oligopeptide systemin (Pearce et al. 1991), diverse oligosaccharides (Bishop et al. 1981), and other phytohormones such as abscisic acid (Pena-Cortes et al. 1989) and ethylene (O'Donnell et al. 1996), as well as physical factors such as hydraulic pressure or electrical pulses, have also been proposed to play a role in wound signaling cascades (Leon et al. 2001).

Enhanced ethylene production is a common occurrence in wounded tissues and is a consequence of the activation of ACC synthase (ACS) and ACC oxidase (ACO), the two ethylene biosynthetic enzymes. Genes encoding specific isoforms of these enzymes are themselves wound inducible (reviewed in Bruxells and Roberts 2001). The induction of various wound-induced defense genes by ethylene (Botella et al. 1996, Rickey and Belknap 1991), suggests that ethylene may be the mechanism for their activation by wounding. In addition to its direct effect on gene expression, ethylene, along with JA, appear to coordinate gene expression. For example, ethylene regulates Pin gene

expression and JA biosynthesis in tomato (O'Donnell et al. 1996), and Arabidopsis leaves (Laudert and Weiler 1998), and is involved in pathogen-resistance mechanisms (reviewed by Bruxells and Roberts 2001), but negatively regulates the expression of a set of JA-responsive genes in wounded Arabidopsis leaves (Rojo et al. 1999) and wound-induced lectin genes in *Griffonia simplicifolia* leaf (Zhu-Salzman et al. 1998). Components of the ethylene signaling pathway have been investigated in Arabidopsis plants (reviewed by Johnson and Ecker 1998). The promoters of a number of ethylene-induced defense genes contain a sequence element termed the ethylene response element (ERE), or "GCC-box"(Johnson and Ecker 1998). The ERE is bound by a class of ethylene-induced transcription factors termed ERE binding proteins (EREPs), or ethylene response factors (ERF) (Ohme-Takagi and Shinshi 1995). Several members of this class of transcription factors are rapidly induced in wounded tobacco leaves in an ethylene- and JA-dependent manner, and their subsequent activation in response to ethylene is required for transcription of defense genes (Suzuki et al. 1998).

JA was originally identified as a potential signal in wounding when its volatile derivative, methyl jasmonate (MeJA), was recognized as a potent inducer of proteinase inhibitor genes in tomato leaves (Farmer and Ryan 1990). MeJA and its free acid JA, collectively referred to as jasmonates, are important cellular regulators mediating diverse developmental processes, such as seed germination, flower and fruit development, leaf abscission, and senescence (reviewed in Creelman and Mullet 1997). Jasmonates induce plant defense responses against a number of pathogens (Reymond and Farmer 1995) and mechanical or herbivorous insect-driven wounding in soybean hypocothyls (Creelman et al. 1992). MeJA has become a strong candidate as an airborne signal providing inter-

plant communication for defense responses (Farmer and Ryan 1990). The biosynthesis of JA is catalyzed by a number of regulated enzymes in several subcellular compartments in reactions collectively known as the octadecanoid pathway (Creelman and Mullet 1997, Leon and Sanchez-Serrano 1999). JA biosynthesis starts when lipid precursors are released from membranes by the action of phospholipases, most probably the plasma membrane or chloroplast membranes. An increase in free linolenic acid in cultured cells of several plant species challenged with fungal elicitors (Gundlach et al. 1992) and in mechanically wounded tomato (Conconi et al. 1996) and castor bean leaves (Ryu and Wang 1996) suggests that the release of linoleic acid from membranes is an important step in controlling JA synthesis. A phospholipase A (EC 3.1.1.4) (PLA)-like activity has been proposed to mediate the release of linolenic acid from membranes (Farmer and Ryan 1992), and a wound-inducible PLA activity has in fact been noted in tomato leaves (Narvaez-Vasquez et al. 1999) and other plant species (Lee et al. 1997). Recently, phospholipase D (EC 3.1.1.4.4) (PLD) has also been shown to be essential for JA synthesis and JA responses in arabidopsis plants (Wang et al. 2000). PLD may generate substrates for PLA, or directly activate lipoxygenases involved in JA synthesis. Many of the genes encoding JA biosynthetic enzymes are induced by wounding and often also by JA, providing wound response feedback control of JA levels (reviewed by Leon and Sanchez-Serrano 1999). It has been reported that a number of biosynthetic intermediates, isomers, derivatives, and metabolites of the octadecanoid pathway are also powerful cellular regulators, depending on biological systems (Hamberg and Gardner 1992, Beale and Ward 1998).

Cloning of the *COI1* gene from an Arabidopsis JA-signaling mutant revealed the mechanism of JA signaling at the molecular level (Xie et al. 1998). The gene encodes an F-box protein, a class of proteins that forms part of a multi-protein complex involved in targeted proteolysis (Xie et al. 1998). The authors suggest that JA-responsive genes are repressed by negative regulatory proteins, which can be specifically degraded following JA perception in a *COI1*- dependent manner leading to the induction of gene expression. The authors further suggested that the induction of gene expression based on the relief of repression rather than on a direct positive induction mechanism, may be common in plants.

Recent studies have suggested that activation of phospholipase D (EC 3.1.4.4) also may play an important role in mediating wound-induced lipid hydrolysis (Ryu and Wang 1996, 1998, Lee et al. 1997). Wang et al. (2000) suggested that multiple forms of PLD were activated in response to wounding in Arabidopsis leaves. Wounding of castor bean leaves rapidly induces PLD-mediated hydrolysis, as evidenced by a rapid accumulation of phosphatidic acid (PA) and choline (Ryu and Wang 1996). Wound-induced PA production has been detected at both the wound site and at sites distal to wounding in castor bean, tomato, soybean, sunflower, broad bean, and pepper (Lee et al. 1997). The activation in castor bean appears to result from translocation of PLD from cytosol to membranes, mediated by an increase in cytoplasmic Ca^{2+} concentration (Ryu and Wang 1996). From analysis of wound activation of PLD and production of various lipid metabolites, Ryu and Wang (1998) proposed a working model to account for the role of PLD in wound responses. According to this model, PLD activation may promote the release of polyunsaturated fatty acids through two processes. First, the PLD-mediated

formation of PA may initiate a lipolytic pathway, involving PLD, PA phosphatase and acyl-hydrolysing enzymes. In this pathway, phospholipids are converted sequentially into PA, diacyl glycerol, and free fatty acids. Consistent with this proposed pathway is that wound-induced PA production in castor bean hypocotyls occurs before DAG and linolenic acid are produced (Ryu and Wang 1998). This PLD-initiated process has also been proposed to occur in deteriorating membranes of senescing and aging plant tissues (Paliyath and Droillard 1992, Samama and Pearce 1993). The second path for the production of free fatty acids involves the PA-induced stimulate of acyl hydrolase or PLA activities. In several plant species, the wound induced PA production occurs before the appearance of lysophosphatidylcholine and lysophosphatidyletanolamine, which could result from PLA activity (Lee et al. 1997). PA has been demonstrated to be an activator of PLA_2 (EC 3.1.1.4) in mammalian systems (Bauldry and Wooten 1997, Kinkaid et al. 1998).

Studies of physically wounded tissues suggest an involvement of polysaccharide degradation in wound metabolism (Esquerre-Tugaye et al. 2000). Huber and Lee (1989) have reported the release of higher amount of pectic oligomers in wounded tomato pericarp compared to intact fruit. The increased production of oligouronides has largely been attributed to PG activity (Huber et al. 2001). PG transcripts and activity have been reported to increase in response to wounding, systemin, and pectic fragments in tomato leaves (Bergey et al. 1999) and fruit (Moretti et al. 1998). The glycan chitosan, a component of fungal cell walls, and fragments derived from the plant cell wall pectins are known to induce *Pin* gene expression in tomato leaves (Bowles 1998). Oligogalacturonic acid (OGA) application to tobacco cell suspension cultures (Mathieu et al. 1991), carrot

protoplasts (Messaiaen and van Cutsem 1994) and tomato leaves ((Thain et al. 1990) results in a rapid depolarization of the plasma membrane and influx of calcium into the cytoplasm, the generation of active oxygen species (Oroczo-Cardenas and Ryan 1999), and the induction of JA (Doares et al. 1995) and ethylene (O'Donell et al. 1996) biosynthesis. In Arabidopsis, OGAs stimulate a class of wound-induced genes expressed predominantly in the local, wounded leaf (Rojo et al. 1999). OGAs induce not only the local wound response genes, but can block the ability of exogenous JA to stimulate the expression of the systemic class of genes (Rojo et al. 1999). The authors have suggested that OGAs released following tissue damage contribute to the induction of locally expressed genes but suppress the systemic wound-response genes. The suppression of the JA response pathway by OGAs is ethylene dependent, and ethylene alone is sufficient to suppress the JA pathway in local (wounded area) leaves in Arabidopsis (Rojo et al. 1999).

Abscisic acid (ABA) is known for its involvement in the regulation of both stomatal closure and dehydration-induced gene expression. There is also evidence of a role for ABA in wound responses, although the exact nature of this role remains unclear. Pena-Cortes et al. (1989) showed that ABA application can directly induce *PinII* gene expression locally and systemically in potato, and to a lesser extent in tomato and tobacco plants. More direct evidence for ABA in mediating the wound response has been reported by Birkenmeier and Ryan (1998), who have shown that ABA application induced *PinII* expression in young tomato plants to a much lesser extent than either wounding or JA application, and further showed that endogenous ABA levels increase significantly in response to wounding only in tissue surrounding the wound site. ABA may also play a

role in inducing dehydration-responsive genes locally following wounding. Reymond et al. (2000) reported that in Arabidopsis leaves dehydration may directly control wound-gene induction. Using DNA microarray technology to analyze the expression of 150 mechanically and insect driven wound-inducible genes, the authors found that many wound-induced genes were also stimulated by dehydration, and the overall profile of wound-induced gene expression was more similar to that induced by dehydration than by insect feeding (Reymond et al. 2000).

One of the consequences of wounding is pathogen ingress and proliferation. Plants have both preexisting and inducible defense mechanisms against invading pathogens. The latter often includes rapid and localized cell death, known as the hypersensitive response, the activation of a complex array of defense genes, and the production of antimicrobial phytoalexins (Yang et al. 1997, Scheel 1998, Somssich and Hahlbrock 1998, Martin 1999). The activation of these defense responses is initiated by the plant recognition of pathogens, either by a gene-for gene-interaction between a plant resistance gene and a pathogen avirulence gene, or by the binding of a non-race specific elicitor such as elicitin to a receptor (Keen 1990, Staskawicz et al. 1995, Hammond-Kosack and Jones 1996, Ricci 1997, Martin 1999). Signals from such interactions are transduced into cellular responses in both host and pathogen (Hammond-Kosack and Jones 1996, Scheel 1998, Martin 1999).

Pharmacological studies using inhibitors of protein kinases and phosphatases suggested an involvement of protein phosphorylation and dephosphorylation in the induction of defense responses including the generation of ROS, activation of defense genes, and hypersensitive cell death (Grosskopf et al. 1990). Increasing evidence

indicates that mitogen-activated protein kinases (MAPK)-like proteins are one of the key regulators in the signaling pathways in plants including tobacco cell suspension cultures and leaves (Romeis et al. 1999), and tomato leaves (Stratmann and Ryan 1997). The MAPK modules function as molecular switches to turn on the expression of genes and cellular responses (Huang and Ferrell 1996). Salicyclic-acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), and their orthologs in several plant species are also thought to be involved in plant defense signaling (Cardinale 2000, Romeis et al. 1999, Kovtun et al. 2000, Nuhse 2000). SIPK was first identified as salicylic acid-induced protein kinase and was later shown to be responsive to a number of biotic and abiotic stresses, including pathogen or pathogen-derived elicitors, ozone, wounding, salt, and osmotic stresses (Hayos and Zhang 2000, Samuel 2000, Zhang and Klessing 2000). The activation of a second MAPK, WIPK, accompanies the activation of SIPK in resistant tobacco suspension cells treated with fungal elicitins (Zheng et al. 2000). The authors have suggested that transient activation of these MAPKs results in various defense responses, and allows the cells to adapt to adverse environments. However, persistent activation of these signal modules leads to apoptosis (Xia et al. 1995, Kyriakis and Avruch 1996, Davis 2000). In tobacco cell suspension cultures, transient activation of SIPK has been linked to L-phenylalanine ammonia lyase gene activation induced by fungal elicitors (Zheng et al. 2000).

The induction of MAPK-like activities is also implicated in the death of tobacco cells treated with xylanase and Arabidopsis cells treated with harpin from *Pseudomonas syringae pv syringae* (Desikan et al. 1996, Suzuki et al. 1999). Inoculation of plant tissues with pathogen, or treatment of cell cultures with microbial elicitors results in an oxidative

burst characterized by the rapid generation of hydrogen peroxide (reviewed in Lamb and Dixon 1997 and Bolwell 1999). Mechanical stimulation of isolated parsley cells (Gus-Mayer et al. 1998) and the treatment of tobacco cell suspension cultures with cell wall-derived OGAs (Legendre et al. 1993, Stennis et al. 1998) also generate H_2O_2 accumulation. H_2O_2 can act as a local signal for hypersensitive cell death and also as a diffusible signal for the induction of defensive genes in adjacent cells (Alvarez et al. 1998).

A model for the expression of defense-related genes in tomato leaves in response to wounding and systemin has been presented (Farmer and Ryan 1992). In this model, systemin initiates a cascade of intracellular events leading to the activation of a cytoplasmic phospholipase that releases linolenic acid from membranes, linolenic acid is converted to JA, a powerful activator of genes coding for both signal pathway enzymes and defensive proteinase inhibitors and polyphenol oxidase (EC 1.10.3.1). The model was altered recently to note that signal pathway genes are expressed within 0.5 h after wounding, whereas defensive genes are expressed within 4 h in tomato leaves (Ryan 2000). PG was demonstrated to be among the early-expressed genes in tomato leaves (Bergey et al. 1999), raising questions regarding its role in the signal transduction pathway, because it was known to produce OGAs from plant cell walls that are activators of both the defensive genes (Ryan and Farmer 1991) and of the production of H_2O_2 in tomato leaves (Stennis et al. 1998). When considering local gene expression, OGAs seem to be the primary elicitors initiating wound responses (reviewed in Bruxells and Roberts 2001), since OGAs are the only elicitors able to mimic all aspects of signaling and the only elicitors known to induce local gene expression in wounded Arabidopsis leaves

(Rojo et al. 1999). In addition to the wound-induced polygalacturonase activity (Rojo et al. 1999), an analysis of the recently completed Arabidopsis genome sequence shows that there are many genes encoding proteins with predicted functions in pectin degradation. Perhaps some of these enzymes are rapidly activated or their catalytic activity is greatly enhanced in response to wounding, releasing OGAs that trigger the wound response.

Systemic signaling requires vascular connections and distribution (Jones et al. 1993, Orians et al. 2000). Some reports have suggested that the systemic signal is carried in the phloem. For example, an electrical signal associated with *Pin* gene expression (Rhodes et al. 1996) and systemin (Narvaez-Vasquez et al. 1994) in tomato are carried by the phloem. Studies on transmissible electrical activity induced by wounding suggested that this electrical activity may be the systemic signal responsible for gene expression in tomato plant (Wildon et al. 1992). The authors showed that the systemic signal generated by mechanical damage was not carried by the phloem. Furthermore, the systemic accumulation of proteinase inhibitors correlated with an electrical signal with the characteristics of an action potential, and applied electrical signals generating action potentials are able to induce *Pin* gene expression in tomato plants (Herde et al. 1995, Stankovic and Davies 1996). Hydraulic signals produced by the release of xylem tension at sites of damage in tomato leaves are also candidates to carry the systemic signal. These hydraulic signals are easily detectable throughout the plant following relatively minor mechanical wounding and even insect feeding in tomato leaves (Alarcon and Malone 1994). The authors have shown a correlation between hydraulic signals and wound-induced gene expression.

Systemin, an 18 amino acid peptide, has also been implicated in systemic signaling (reviewed by Ryan 1998). Although no definitive conclusion has been reached that systemin is mobile, it is central to the expression of wound-induced genes and is required for systemic signaling in tomato plants. In tomato plants, systemin comprises the C-terminal region of a much larger precursor, known as prosystemin (McGurl et al. 1992). The prosystemin gene is expressed primarily in the vascular regions of the aerial parts of the plant and is itself wound inducible. Transgenic tomato plants in which prosystemin gene expression is suppressed via antisense RNA expression exhibit significantly reduced systemic *Pin* gene expression in response to wounding (McGurl et al. 1992). Transgenic plants over-expressing prosystemin under the control of the constitutive CaMV 35S promoter show high levels of *Pin* gene expression throughout the plant (McGurl et al. 1994). Experiments with synthetic systemin analogues containing amino acid deletions and substitutions previously showed that the entire peptide is required for full biological activity and that the four C-terminal residues are the most important in inducing wound gene expression (Pearce et al. 1993). While the C-terminal region of systemin is required for its activity, the N-terminal region appears to be essential for the interaction with its receptor (Meindl et al. 1998).

Volatile signals have been reported to play roles in plant defense enabling plants to form structural barriers to potential attackers and to store toxic compounds, and to synthesize and activate defensive proteins and metabolites (reviewed in Bruxelles and Roberts 2001). Although the research regarding the role of plant volatiles in plant defense has largely been focused on tritrophic interactions involving plants, herbivores and their

natural enemies, plant volatiles also provide interplant protection against pathogens (Shualev et al. 1997).

Lipases

Lipoxygenases

Lipoxygenase (EC 1.13.11.12, LOX) represents a large gene family of nonheme iron fatty acid dioxygenases, which are ubiquitous in plants (Brash 1999). They catalyze the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids (PUFA) containing a (1Z,4Z)-pentadiene system (Feussner and Kuhn 2000). Plant LOXs are classified based on their positional specificity of linolenic acid (LA) oxygenation, which is oxygenated either at carbon atom 9 (9-LOX) or at C-13 (13-LOX) of the hydrocarbon backbone of the fatty acid leading to two groups of compounds, the (9S)-hydroperoxy- and the (13S)-hydroperoxy-derivatives of PUFAs (Feussner and Wasternack 2002).

With respect to overall sequence similarity, Feussner and Wasternack (2002) have grouped plant LOXs into two gene subfamilies. Type 1-LOXs display no transit peptide and have a high sequence similarity (>75%) to one another. Type 2-LOXs possess a putative chloroplast transit peptide sequence and demonstrate a moderate overall amino acid sequence similarity (~ 35%) to one another. Plant LOXs are multifunctional enzymes, catalyzing at least three different types of reactions: (a) dioxygenation of lipid substrates; (b) secondary conversion of hydroperoxy lipids; (c) formation of leukotrienes (Shimizu et al. 1984). Dioxygenation of lipid substrates is the most common function in plants. A hypothesis for a plant LOX reaction mechanism has been suggested in soybean seeds (Gardner 1989). According to this hypothesis, substrate orientation is the key step in the determination of the position of dioxygen insertion which then leads to varying regiospecificities of different isozymes: In the case of 13-LOXs, the active site is

penetrated by the substrate using its methyl end first. Whereas with 9-LOXs, the substrate is forced by enzyme into an inverse orientation favoring a penetration with its carboxy group first (Gardner 1989). Consequently, a radical rearrangement at either (+2) or (-2), respectively may be facilitated in both cases. Most plant LOXs strongly prefer free fatty acids as substrates (reviewed in Siedow 1991). Two 13-LOXs reported from soybean seeds (Brash et al. 1987) and cucumber roots (Matsui et al. 1998), however, have been shown to act on PUFAs esterified to phospholipids. Additionally, LOX activity against PUFAs esterified in neutral lipids such as triglycerides has been shown in cucumber cotyledons (Feussner et al. 1998) and soybean seeds (Fuller et al. 2001).

LOXs are believed to play a role in many developmental processes. The involvement of *AtLOX2* in JA biosynthesis upon wounding was shown using arabidopsis plants expressing an antisense construct for *AtLOX2* (Bell et al. 1995). These plants were unable to accumulate JA upon wounding, and the expression of JA-and wound-inducible genes such as *vsp* was significantly reduced. In potato leaves, the type 2 13-LOXs, LOX-H3 and LOX-H1 are induced upon wounding (Royo et al. 1996). Certain LOXs have been implicated in conferring resistance against pathogens (reviewed in Slusarenko 1996). Expression of *a 9-LOX* gene in tobacco and potato provided resistance against *phytophytora* (Rance et al. 1998). Additionally, the suppression of *9-LOX* gene in potato has been reported to result in reduced tuber yield and size, and to disrupt tuber formation, showing that 9-LOX-generated metabolites are involved in the regulation of tuber development (Kolomiets et al. 2001). Among eight different LOX forms identified in soybean, some are found in paraveinal mesophyll cells and are believed to function as storage proteins (Grimes et al. 1993).

Phospholipase C

Phospholipase C (EC 3.1.4.3) (PLC) cleaves the glycerophosphate ester linkage of phospholipids generating DAG and phosphorylated groups (Wang 2001). Based on substrate specificity and cellular function, PLCs in plants are classified into three classes: (a) PI-PLC that hydrolyzes phosphoinositides, (b) the nonspecific-PLC hydrolysing phosphatidylcholine (PtdCho) and some other phospholipids and (c) the glycosylphosphatidylinositol GPI-PLC acting on GPI-anchors on proteins (Wang 2001). Several PLCs hydrolyzing PtdCho have been cloned and characterized from gram positive and gram negative bacteria (reviewed in Titball 1998). The Arabidopsis Genome Project has revealed six Arabidopsis genes demonstrating sequence similarities to bacterial PLCs; however, the catalytic properties of these proteins have not yet been verified experimentally. A GPI-PLC hydrolysing only solubilized GPI-anchor was partially purified from peanut seeds (Butikoffer and Broadbeck 1993), but no GPI-PLC activity has been characterized in plants (Wang 2001). PI-PLC has been cloned from several plants including arabidopsis vegetative and floral tissues (Hartweck et al. 1997, Hirayama et al. 1997) and potato tubers (Kopka et al. 1998). These PLCs possess domains X (~ 170 amino acids) and Y (~260 amino acids), essential for phosphoesterase activity, followed by a Ca^{2+}-dependent phospholipid binding C2 domain toward the C terminus region (reviewed in Wang 2001). Ca^{2+} is needed for plant PI-PLC activities regulating PI-PLC via catalysis and membrane binding in *Catharantus roseus* cells (Hernandez-Sotomayor et al. 1999). The cloned PI-PLCs form potato tubers favor phosphatidylinositol diphosphate as a substrate at physiological concentrations of Ca^{2+} (μM), but prefers PtdIn at millimolar levels of Ca^{2+} (Kopka et al. 1998). PI-PLC activity is also modified by Mg^{2+} and Al^{3+} (reviewed in Wang 2001). Based on substrate

specificities and cation effects, it is indicated that the cellular activity of PI-PLC is modified by cellular location, association with other cellular factors, and membrane environments (Pappan et al. 1998, Wang and Dennis 1999, Wang 2000).

PI-PLC-mediated signaling is important in plant responses to various stimuli, comprising osmotic stress, ABA, light, gravity, pathogen attack, and pollination (Wang 2001). It involves in the light-dependent regulation of C4 phosphoenolcarboxylase through phosphorylation of the enzyme (Coursol et al. 2000). PI-PLC generates important cellular messengers including inositol 1,4,5 triphosphate (InP3), and DAG (reviewed in Wang 2001). InP3 was demonstrated to induce an increase in cytoplasmic Ca^{2+}, and to attenuate the inward K^+ channel of the plasma membrane (Staxen et al. 1999). DAG is an important activator of protein kinases. Additionally, the activity of PI-PLC reduces the membrane levels of PtdIn(4,5)P2 , which is an activator of PLD, a substrate for PI-3 kinase, an attachment site for various proteins, and is required for membrane trafficking events (reviewed in Wang 2001).

Phospholipase D

Phospholipase D (EC 3.1.4.4) (PLD) hydrolyses phospholipids at the terminal phosphodiesteric bond, generating phosphatidic acid and water-soluble free groups such as choline (Wang 1999). Based on the requirements for Ca^{2+} and lipids in vitro assays, PLDs were classified into three groups: (a) the traditional PLD, most active at millimolar levels of Ca^{2+} (20 to 100 mM), (b) the polyphosphoinositide (PI)-dependent PLD, most active at micromolar levels of Ca^{2+}, (c) the phosphatidylinositol (PtdIn)-specific PLD, shown to be Ca^{2+} independent (reviewed in Wang 2000). The traditional PLD purified from several plant sources (reviewed in Wang 2000) is the most widespread and best studied class in plants. The PI-dependent PLD was characterized in arabidopsis leaves

(Pappan et al. 1997, Qin et al. 1997, Wang 1999). The PtdIn-specific PLD was identified in suspension cells of *Catharantus roseus* (Wissing et al. 1996). Based on the deduced amino acid sequence similarities, gene architecture, and biochemical properties, PLDs in Arabidopsis are divided into five groups, PLDα, β, γ, δ, and ε (Wang 2001). Most of the PLDs cloned from other plant species belong to the PLDα group, and multiple PLDαs have been cloned from cabbage (Kim et al. 1999). PLDα is the conventional plant PLD and is PI-independent when assayed at millimolar concentrations of Ca^{2+} (Pappan et al. 1997). In contrast, PLDβ and PLDγ require a PI cofactor and are most active at micromolar concentrations of Ca^{2+} (Pappan et al. 1997, Qin et al. 1997).

The PLDs cloned from all eukaryotes possess two HxKxxxD motifs, which form two active-site regions required for PLD activity (Xie et al. 2000). The PLD superfamily is defined by the presence of this motif. The determination of the crystal structure of PLD revealed the mode of action of the enzyme (Stucky and Dixon 1999). The enzymes of the PLD superfamily utilize the conserved histidine for nucleophilic attack on the substrate phosphorus. PLD cleaves phospholipids at the P-O rather than the C-O bond through a two-step Ping Pong reaction mechanism involving a phosphatidylated enzyme intermediate (Stucky and Dixon 1999).

PLD is involved in various regulatory processes, such as those leading to hormone action (Fan et al. 1997, Jacob et al. 1999), cell proliferation (Daniel et al. 1999), membrane trafficking, secretion (Colley et al. 1997, Jones et al. 1999), and defense responses (Waite et al. 1997, Wang 1999). Specifically, PLD plays important roles in plant responses to stresses, such as through mediating the action and production of the stress-related hormones, ABA in arabidopsis guard cells (Jacob et al. 1999), JA in

arabidopsis leaves (Wang et al. 2000) and ethylene in carrot suspension cells (Lee et al. 1998). PLD contributes significantly to wound-mediated lipid hydrolysis. Wang et al. (2000) suggested that multiple forms of PLD were activated in response to wounding in Arabidopsis leaves. Wounding of castor bean leaves rapidly induces PLD-mediated lipid hydrolysis, as evidenced by a rapid accumulation of PA and choline (Ryu and Wang 1996).

CHAPTER 3
ACTIVITIES OF SEVERAL MEMBRANE AND CELL-WALL HYDROLASES, ETHYLENE BIOSYNTHETIC ENZYMES, AND CELL WALL POLYURONIDE DEGRADATION DURING LOW-TEMPERATURE STORAGE OF INTACT AND FRESH-CUT PAPAYA (*CARICA PAPAYA*) FRUIT

Introduction

Fresh-cut fruits and vegetables are those that have been subjected to various degrees of peeling, trimming, coring, slicing, shredding or dicing. The objectives of fresh-cut technology are to deliver to consumers a convenient, fresh-like product with extended shelf life, and high nutritional and sensory quality (Reyes 1996).

Loss of protective epidermal and subepidermal tissues, and acute physical injury associated with processing, contribute to the high perishability of fresh-cut produce (Reyes 1996). Fresh-cut tropical fruits including banana (Abe and Watada 1991), and papaya (O'Connor-Shaw et al. 1994; Paull and Chen 1997), for example, were of unacceptable quality after only 2 days storage at 4 °C, primarily due to tissue softening. The biochemical basis of texture loss in fresh-cut fruits is unknown. In view of the physical damage associated with fruit processing, likely candidates for rapid metabolic responses to wounding include the cell wall and membranes. The activities of some cell wall enzymes, for example, are minimal, if any in intact tissue, but are greatly enhanced in response to mechanical wounding (Dumville and Fry 2000, Huber et al. 2001). The senescence-delaying influence of Ca^{+2} dips on shredded carrots (Picchioni et al. 1996) and the increased juice leakage during storage of fresh-cut watermelon (Cartaxo et al.

1997) are consistent with an important role for altered cell wall and/or membrane metabolism in the deterioration of fresh-cut produce.

Although fresh-cut commodities initially possess the quality and sensory attributes of the intact commodity, the metabolism of fresh-cut compared with intact fruit diverges rapidly. Fresh-cut products show enhanced respiration and ethylene production, and are significantly more perishable than the intact commodity (Varaquaux and Wiley 1994). Several studies have emphasized a role for specific enzymes in the deterioration of fresh-cut commodities. Enhanced activities of chlorophyll-degrading enzymes in cole slaw (Heaton et al. 1996) and pyrophosphatase-phosphofructokinase in sliced carrots (Kato-Noguchi and Watada 1996) have been reported. A wound-induced increase in phenylalanine ammonia lyase was associated with browning of the cut surfaces of head lettuce (Lopez-Galvez et al. 1996) and carrot roots (Leja et al. 1997). Wound-induced phospholipase A_2 in tomato (Narvaez-Vasquez et al. 1999), phospholipase D in Arabidopsis and lipoxygenase in tomato (Heitz et al. 1997) has been shown to be essential for jasmonic acid (JA) synthesis, a wound signal, and JA responses (Wang et al. 2000). During the first hours after wounding, plants accumulate phosphatidic acid and unesterififed fatty acids that are released from lipids presumably by the action of wound-inducible phospholipases of type D and A_2 (Conconi et al. 1996, Lee et al. 1997, Narvaez-Vasquez et al. 1999, Ryu and Wang 1996) thus producing substrates for lipoxygenases (de Bruxelles and Roberts 2001). Limited studies have addressed enzymes contributing to deterioration of fresh-cut fruit, especially those with high chill sensitivity.

The objectives of this study were to determine changes in the activities of several catabolic and wound-induced enzymes, including ethylene biosynthetic enzymes, and in

cell wall polyuronides in fresh-cut papaya fruit stored at low temperature. To distinguish between changes induced by wounding versus those induced by exposure to low-temperature, intact papaya fruit stored under identical conditions were included as controls.

Materials and methods

Plant material and processing procedures

Papaya (*Carica papaya*, var. Sunrise Solo) fruit were obtained from Brooks Tropicals, Homestead, FL, USA. Following transfer to the postharvest facilities in Gainesville, the fruit were sorted, washed with tap water, chlorinated water (150 μL L^{-1} free chlorine, pH 7.0) and rinsed. The fruit were then stored at 20 °C until they reached 60 to 70% yellow surface color. Prior to slicing, the cutting surfaces and cold room interior were rinsed with chlorinated water (150 μL L^{-1} free chlorine). Hairnets, latex gloves, surgical masks, and disposable aprons were worn during cutting and handling to minimize contamination. All operations were performed at 5 °C and fruit were held at this temperature for 12 h prior to processing. Fruit were peeled, and tissue processed into pieces (~7 cm x 5 cm x 3 cm) with sharp, sterile knives. Knives were re-sterilized in 150 μL L^{-1} free chlorine during the cutting operations. The tissue pieces were randomized and stored in vented plastic containers for 8 days at 5 °C. Intact fruit stored under identical conditions served as controls.

At the indicated intervals, fruit pieces were removed from storage, immediately frozen in liquid nitrogen, and analyzed as described below. Intact fruit were peeled, cut into pieces as described above, and immediately frozen in liquid nitrogen. Samples were stored at -30 °C until analyzed.

Firmness of fresh-cut and intact papaya

Twenty cubes of approximately 9 cm^3 were excised from the intact and fresh-cut papaya mesocarp tissue pieces and firmness of individual cubes was measured using an Instron Universal Testing Instrument (Model 4411, Canton, MA, USA). After establishing zero force contact with the cube, a 10-mm diameter convex probe was driven (crosshead speed 10 cm/min, 5 kg load cell) a distance of 2 mm. The firmness data represent the maximum force (N) recorded during compression at 5 °C.

Preparation of ethanol-insoluble solids

Partially thawed tissue (100 g) derived from fresh-cut and intact papaya fruit at each sampling interval was homogenized in 400 mL ethanol for 2 min with a Polytron homogenizer (Brinkmann, PT 10-35, Lens Kruenz, Switzerland) set at maximum speed. The homogenate was refluxed in a boiling water bath for 25 min to inactivate enzymes and then filtered through GF/C filter paper (Whatman Inc., Clifton, NJ, USA) in an aspiration flask and washed with 95% ethanol. The residue was transferred to 100 mL chloroform/methanol (1:1, v/v) and incubated with stirring for 30 min. The suspensions were filtered (GF/C) and washed with 100 mL acetone. After partial drying via aspiration, the ethanol insoluble solids (EIS) were placed in a drying oven at 43 °C for 12 h and stored in a desiccator at room temperature.

Extraction and chromatography of water and chelator-soluble polyuronides

Extraction of water-soluble polyuronides was performed by incubating EIS (20 mg) in 7 mL of deionized water for 4 h at room temperature with stirring. The suspension was filtered through GF/C filter paper and the supernatant retained for gel chromatography. The retained material was suspended in 7 mL of 50 mM Na-acetate, 50 mM *trans* -1,2-cyclohexanediamine- *N, N, N', N'* - tetraacetic acid (CDTA), pH 6.5 and incubated at

room temperature with stirring for 6 h. The suspension was filtered through GF/C filter and the supernatant retained for chromatography. Uronic acids in the filtrates were determined using the procedure of Blumenkrantz and Asboe-Hansen (1973). Total uronic acids in the EIS were measured as described in Ahmed and Labavitch (1977).

Gel chromatography of polyuronides was performed as described by Chun and Huber (2000) on a bed (1.5 cm width, 27 cm length) of CL-4B-200 (Pharmacia, Piscataway, NJ, USA) packed and operated in 200 mM ammonium acetate, pH 5.0. Polyuronides (~ 0.5 mg galacturonic acid equivalents in a volume of 2.5 mL) were applied to the column and eluted with ammonium acetate. Fractions of 2 mL were collected, and 0.5 ml aliquots of these used for the determination of uronic acids. Uronic acids from each treatment were chromatographed in triplicate. The V_o and V_i were determined using Dextran 2,000 and glucose (Sigma, St. Louis, MO, USA), respectively.

Isolation and assay of cell-wall enzymes

Partially thawed mesocarp tissue (20 g) derived from fresh-cut and intact papaya fruit and 40 ml cold ethanol were homogenized for 2 min with a Polytron homogenizer, and the homogenate was centrifuged for 20 min (4 °C) at 12,000 x *g* (Beckman, Model J2-21, Palo Alto, CA, USA). The pellet was washed with 25 mL of 80% cold ethanol and centrifuged at 12,000 x *g* (4 °C) for 20 min. The pellet was suspended in 10 mL of 25 mM Na-acetate, pH 5.0 containing 1.2 M NaCl and incubated at 1 °C for 30 min. After centrifugation at 12,000 x *g* (4 °C) for 20 min, the cell-free protein extract was filtered through Miracloth (Calbiochem, La Jolla, CA, USA) and used for the determination of polygalacturonase (EC 3.2.1.15), and α- (EC 3.2.1.22) and β-galactosidase activities (EC 3.2.1.23). Total protein was determined using a standard BCA kit (Pierce Chem. Co., Rockford, IL, USA) with BSA as standard.

Reaction mixtures for the determination of PG activity consisted of 0.1 mL of the cell-free protein extract and 0.5 mL (1 mg) of polygalacturonic acid (citrus, from Sigma) in 50 mM Na-acetate, pH 5.5. The samples were incubated for 2 h in a water bath at 34 °C. Activity was assayed reductometrically (Milner and Avigad 1967), and expressed as mol galacturonic acid equivalents (kg protein)$^{-1}$ h^{-1}.

Reaction mixtures for α- and β-galactosidase activities contained 0.2 mL of the cell-free protein extract and 0.2 mL of the ρ-NO_2-phenyl derivatives (6.6 mM) of α- and β-D-galactopyranoside (Sigma) in 0.1 M Na-acetate, pH 5.2. The reactions were carried out for 20 min at 37 °C and terminated by addition of 1 mL of 1 N NH_4OH containing 2 mM EDTA. The release of ρ-NO_2-phenol was measured at 400 nm. ρ-NO_2-phenol (Sigma) was used as standard. Activity was expressed as mol ρ-NO_2 phenol (kg protein)$^{-1}$ min^{-1}.

Pectinmethylesterase (PME, EC 3.2.1.11) was extracted and assayed as described by Hagerman and Austin (1986). Activity was expressed as mol ^{+}H equivalents (kg protein min)$^{-1}$

ACC synthase activity

ACC synthase activity (EC 4.4.1.14) was determined as described by Mullins et al. (2000) with slight modifications. Tissue (5 g) derived from fresh-cut or intact papaya fruit was homogenized with a Polytron homogenizer in a buffer containing 100 mM potassium phosphate, pH 8.0, 5% ammonium sulfate, 5 μM pyridoxal phosphate, 4 mM dithiothreitol (DTT) and 3% polyvinylpolypyrrolidine (PVPP). The extract was filtered through a layer of Miracloth (Calbiochem) and centrifuged at 10,000 x g for 10 min. The supernatant was brought to 90% saturation with solid ammonium sulfate, stirred for 90 min at 4 °C and the suspension centrifuged at 25,000 x *g* for 10 min. The pellet was

resuspended in 5 mL of incubation buffer (100 mM potassium phosphate, 0.1 mM DTT and 4 μM pyridoxal phosphate, pH 7.0). For desalting, the sample was dialyzed against incubation buffer overnight. ACC synthase activity was determined in a reaction mixture containing 3.67 mL desalted enzyme extract, 375 μL incubation buffer and 450 μL of 500 μM S-adenosyl-L-methionine (AdoMet). After 3 h at 30 °C, protein was eliminated from the mixture by adding 3.75 mL of phenol/chloroform/ isoamyl alcohol (25:24:1) followed by vortexing and then centrifuging for 10 min at 28,500 x *g*. The aqueous phase was decanted and clarified by centrifuging at 28,500 x g for 10 min. ACC formed was assayed by the method of Lizada and Yang (1979) using 1 μmol of $HgCl_2$ and 1 mL of headspace gas for injection into the GC. Activity was expressed as mol of ACC formed $(kg\ protein)^{-1}\ h^{-1}$.

ACC oxidase activity

ACC oxidase was extracted and assayed as described by Fernandez-Maculet and Yang (1991), with some modifications. Four g of papaya tissue were homogenized in 12 mL of extraction medium containing 0.1 M Tris (pH 7.4), 10% glycerol and 30 mM sodium ascorbate. The slurry was filtered through four layers of cheesecloth and centrifuged at 28,000 x g for 25 min. Enzyme activity was assayed at 25 °C in 1 mL reaction mixtures containing 0.4 mL of the enzyme extract, 30 mM sodium ascorbate, 0.1 mM $FeSO_4$, 1 mM ACC and extraction buffer in 25 mL vials stoppered with rubber septa. The reaction mixtures were incubated at 25 °C for 2 h, and 1 mL of gas sample withdrawn from headspace was analyzed for ethylene by gas chromatography. ACC oxidase activity was expressed as mmol ethylene formed $(kg\ protein\ h)^{-1}$.

Lipoxygenase and phospholipase C and D activities

Lipoxygenase activity (EC 1.13.11.12) was determined as described by Cherif et al. (1997). Phospholipase D (PLD) (EC 3.1.4.4) and phospholipase C (PLC) (EC 3.1.4.3) were extracted as described by Ryu and Wang (1996) with some modifications. Briefly, papaya mesocarp tissue (10 g) was homogenized at 4 °C in 10 mL of extraction buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M sucrose, 10 mM KCl, 1 mM EDTA, 0.5 mM PMSF and 2 mM DTT. The homogenate was centrifuged at 15,000 x g for 30 min, and the supernatant used for enzyme assay. PLC and PLD activities were determined spectrophotometrically as described by Kurioka and Matzuda (1976) and Gupta and Wold (1980), respectively, using ρ-nitrophenylphosphorylcholine (Sigma) as substrate. Activity was expressed as mol ρ-nitro-phenol (kg protein h)$^{-1}$.

Statistical analysis

The data were analyzed according to a completely randomized design using GLM program of Statistical Analysis System (SAS) and Duncan's Multiple Range Test at 5% level of significance.

Results and discussion

Firmness of fresh-cut papaya fruit decreased nearly 36% after only 2 d and continued a steady decline throughout storage at 5 °C (Table 3-1). In contrast, tissue derived from intact fruit stored under identical conditions showed little change in firmness through 4 d of storage. At 8 d, the firmness of tissue from fresh-cut and intact fruit had declined approximately 54 and 19%, respectively. The rapid reduction in fresh-cut papaya is consistent with other reports (O'Connor-Shaw et al. 1994, Paull and Chen 1997).

Total polyuronide content did not change in tissue of intact papaya stored for up to 8 d at 5 °C, whereas a small but significant decline (10%) was noted for the fresh-cut fruit (Table 3-1). In intact fruit, levels of chelator-soluble polyuronides did not change whereas water-soluble polyuronides showed a significant increase after 8 d at 5 °C (Table 3-1). In fresh-cut fruit, the levels of both water- and CDTA-soluble polyuronides increased significantly within the first day and continued to increase with storage. After 8 d, chelator- and water-soluble polyuronides in fresh-cut papaya were 30% and 45% higher, respectively, compared with levels at day 0 (Table 3-1). Chelator- and water-soluble polyuronides from intact fruit showed little change in mol mass during storage (Fig. 3-1). Mol mass downshifts in polyuronides in fresh-cut tissue were first evident at 2 d of storage (not shown), with more extensive downshifts evident at 4 and 8 d (Fig. 3-1).

The lower mol mass polyuronides from fresh-cut compared with intact fruit could arise from depolymerization or from increased solubility (Table 3-1) of inherently smaller polymers. Consistent with the participation of depolymerization were the significantly greater levels of PG activity in fresh-cut compared with intact fruit (Fig. 3-2). Paull et al. (1999) have attributed the mol mass downshifts in papaya fruit during ripening to increases in PG activity. Further evidence for polyuronide depolymerization in fresh-cut fruit was the decline in total uronic acids (Table 3-1), suggesting that a portion of polyuronides was converted to ethanol-soluble products (oligouronides) that would not be recovered in EIS preparations. The higher levels of PG activity in fresh-cut fruit might represent a response to increased ethylene production, which has been shown to increase as much as 10-fold in papaya fruit within hours of slicing (Paull and Chen 1997). Ethylene-induced transcriptional activation of PG synthesis has been demonstrated

for tomato (Sitrit and Bennett 1998) and avocado (Buse and Laties 1993, Dopico et al. 1993) fruits. Consistent with a possible role for ethylene in the increases in PG activity, ACC synthase (ACS) and ACC oxidase (ACO) activities increased markedly in fresh-cut and intact papaya (Fig. 3-3A, B). After 2 d of storage, levels of ACS activity in fresh-cut and intact fruit were 250% and 63% higher, respectively, compared with those of fruit prior to storage (Day 0). The differences in ACO activity between fresh-cut and intact fruit (Fig. 3-3 B) were not as dramatic as noted for ACS. ACO activity in intact fruit reached a maximum at Day 2 (a 31% increase compared with Day 0) and then decreased significantly with further storage. In fresh-cut tissue, maximum activity was at Day 4, at which time the activity was 47% higher than the activity at Day 0. Despite the differences in timing and maximum activities between intact and fresh-cut papaya, ACO activity does not show a clear pattern with fresh-cut in papaya and is probably not rate limiting at the activity levels observed (Lelievre et al. 1997).

Other enzymes involved in pectin metabolism and depolymerization, including galactosidases and pectinmethylesterase (PME), were also examined in fresh-cut papaya. PME was not different in fresh-cut compared with intact fruit (Fig. 3-4), with activity increasing through 2 d of storage in both tissues and thereafter remaining constant. Although pectin de-esterification declines during papaya ripening (Paull et al. 1999), our data suggest that levels of extractable PME do not parallel the softening and deterioration of fresh-cut compared with intact papaya fruit. Analysis of pectin mol mass in tomato fruit homogenates, however, provides evidence that pre-existing levels of enzymes (PG and PME) may be sufficient to explain the rapid (within 5 min) and extensive depolymerization in response to tissue disruption (Huber et al. 2001).

Alpha- (α-Gal) and beta-(β-Gal) galactosidase activities were enhanced in fresh-cut compared with intact papaya (Fig. 3-5). In fresh-cut tissue, α- and β-Gal activities increased 86 and 76%, respectively, after only 24 h compared with Day 0 fruit. After 4 d of storage, activity levels remained significantly higher in fresh-cut tissue than levels in intact fruit. Over the entire storage period, α- and β-gal activities increased 147% and 116%, respectively, in fresh-cut fruit compared with 18% and 64% in intact fruit. Over the 8-day storage period, the enhanced activity due to fresh-cut (wounding) was 83% and 33%, respectively, for α- and β-Gal. As for PG, the increased activities of galactosidases might represent a response to enhanced ethylene production. Galactosidase activities were suppressed in ACS-antisense tomato fruit, which had greatly reduced ethylene production, but accumulated in fruit exposed to exogenous ethylene (Sozzi et al. 1998). Although the functions of galactosidases in pectin metabolism in particular and cell wall metabolism in general are not fully understood (Huber et al. 2001), the trends in the activities of both enzymes paralleled the firmness declines in the fresh-cut and intact papaya. A correlation between α- and β-Gal activities increases and firmness decline has been reported for ripening papaya fruit (Lazan and Ali 1993, Lazan et al. 1995). Furthermore, Tucker et al. (1999) reported that cold-break pastes from tomato fruit expressing an antisense-gene for a β-galactanase exhibited higher viscosity compared with pastes from normal fruit, providing indirect evidence of a role for these enzymes in the degradation of pectins, and possibly other structural polymers.

Another factor of potential importance in the rapid softening and deterioration of fresh-cut fruit is increased cellular leakage (Cartaxo et al. 1997, Lopez-Galvez et al. 1997, Hodges et al. 2000) resulting from wound-induced degradation of membrane lipids.

An increase in electrolyte efflux in response to cutting has been noted for papaya fruit (Ergun and Huber unpublished). Wu et al. (1999) reported that increased lipoxygenase (LOX) activity was associated with firmness loss in peach mesocarp tissue. As shown in Fig. 3-6, LOX activity increased dramatically in response to cutting, increasing more than 3 fold in the initial 24 h of storage and reaching a maximum at Day 4. In contrast, LOX activity increased slightly in intact fruit during storage. PLC activity increased markedly (32%) in fresh-cut fruit within 24 h (Fig. 3-7). A parallel but delayed increase was also noted for intact papaya on Day 2. After Day 2, PLC activity decreased in both fresh-cut and intact tissue through 8 d of storage. PLD activity in fresh-cut tissue increased (48%) within 24 h and then remained relatively constant during the rest of the storage period. However, PLD activity increased significantly within 4 d in intact fruit and then remained constant throughout the storage (Fig. 3-7). Generally, the activity of lipolytic enzymes including phospholipases and LOXs increases during senescence (Todd et al. 1990, Wang 2001), the former activities resulting in the release of membrane unsaturated fatty acids that can serve as substrates for LOX. In response to physical wounding, LOX could be involved either positively, through its role in the production of defense-related signaling molecules (Creelman et al. 1992, Albrecht et al. 1993, Laudert et al. 1996), or negatively through participation in autocatalytic peroxidation reactions (Hidelbrand 1989). LOX hydroperoxides can contribute to tissue damage through inactivation of protein synthesis and deterioration of cellular membranes. Dumville and Fry (2000) and Schweikert et al. (2000) have posited a role for radical-based mechanisms in polysaccharide breakdown in plant development, providing a possible relationship between peroxidative lipid metabolism and tissue softening.

In summary, fresh-cut and intact papaya stored at 5 °C showed dramatic differences in tissue firmness and in the activity trends of a number of hydrolases. The generally higher and more rapid accumulation of enzyme activities in fresh-cut tissue might represent a general or global response to wounding. The rapidity of these increases, typically observed within 24 h of tissue wounding, argues that microbial proliferation, which becomes more problematic during long-term storage of fresh-cut fruit (Cartaxo et al. 1997), did not contribute to the enhanced activities of cell wall and membrane hydrolases. Furthermore, the differences noted between intact and fresh-cut fruit, which were stored under identical conditions, support the notion that the firmness and enzyme trends in fresh-cut papaya are not a direct consequence of low-temperature stress. The rapid softening and deterioration of fresh-cut papaya likely involve membrane and cell wall catabolism accelerated or otherwise altered in response to physical wounding. Wound-induced changes in permeability and solute efflux could modify Ca^{+2} binding (Ferguson et al. 1980) and other apoplastic conditions (Almeida and Huber 1999), contributing to accelerated cell wall and membrane catabolism. Enhanced polyuronide degradation in response to tissue damage (Brummell and Labavitch 1997, Fry 1998, Huber et al. 2001), for example, is consistent with a role for wound-induced modification of apoplastic conditions in the deterioration of fresh-cut fruits. Collectively, the data suggest that enhanced hydrolase activity and accelerated senescence are involved in the rapid softening and deterioration of fresh-cut papaya fruit.

Table 3-1. Changes in firmness and cell-wall polyuronides of fresh-cut and intact papaya fruit during storage at 5 °C.

	Firmness (N)		Total Polyuronides (g/kg)		Water-soluble Polyuronides (g/kg)		CDTA-soluble Polyuronides (g/kg)	
Days	Intact	fresh-cut	Intact	fresh-cut	Intact	fresh-cut	Intact	fresh-cut
0	8.7a	-----	327.0a	-----	62.3b	-----	34.7a	-----
1	8.7a	7.8a	326.1a	325.5a	64.2ab	72.7b	35.1a	40.9b
2	8.5a	5.6b	328.7a	321.1a	68.0ab	75.1b	36.1a	44.5ab
4	8.1a	5.0bc	327.1a	312.1a	68.5ab	77.1ab	36.7a	46.7ab
8	7.0b	4.1c	321.4a	295.2b	71.7a	83.1a	37.0a	50.3a

Means followed by the same letter within each column are not significantly different at the 5% level.

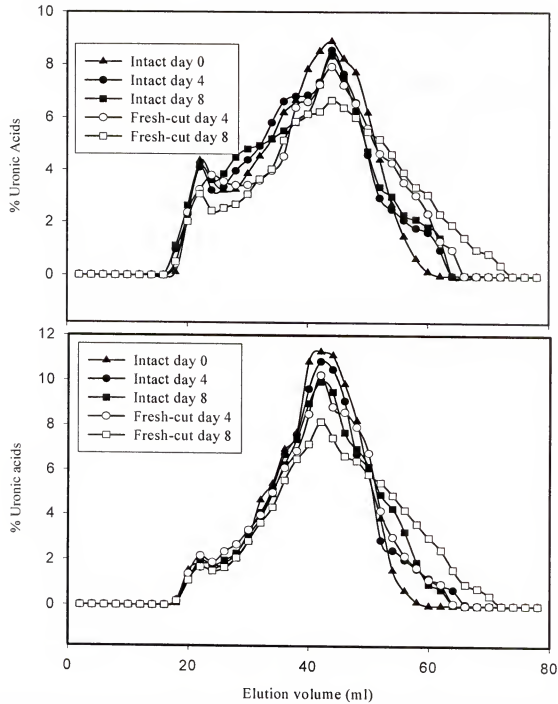

Figure 3-1. Mol mass profiles of CDTA- (A) and water-soluble (B) polyuronides from intact and fresh-cut papaya fruit stored at 5 °C for 0, 4 and 8 d. Polyuronides (0.5 mg galacturonic acid equivalents) were applied to a CL-4B-200 (1.5 x 27 cm) column operated with a mobile phase of 200 mM ammonia acetate, pH 5.0. Fractions were analyzed for uronic acids. V_o and V_i denote the void and included volumes, respectively.

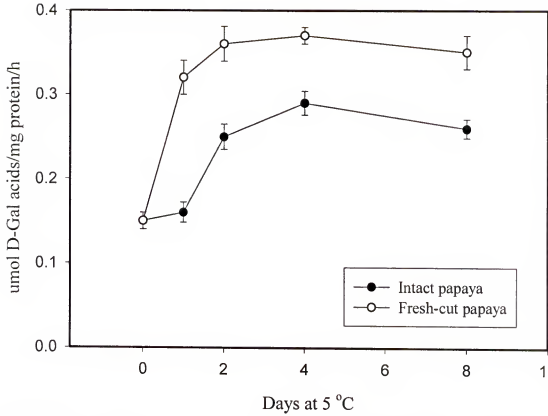

Figure 3-2. Polygalacturonase activity (D-Gal per kg of protein) of intact and fresh-cut papaya fruit stored at 5 °C for 0 1, 2, 4 and 8 days. Data are the means of 6 replications. Vertical bars represent standard deviation.

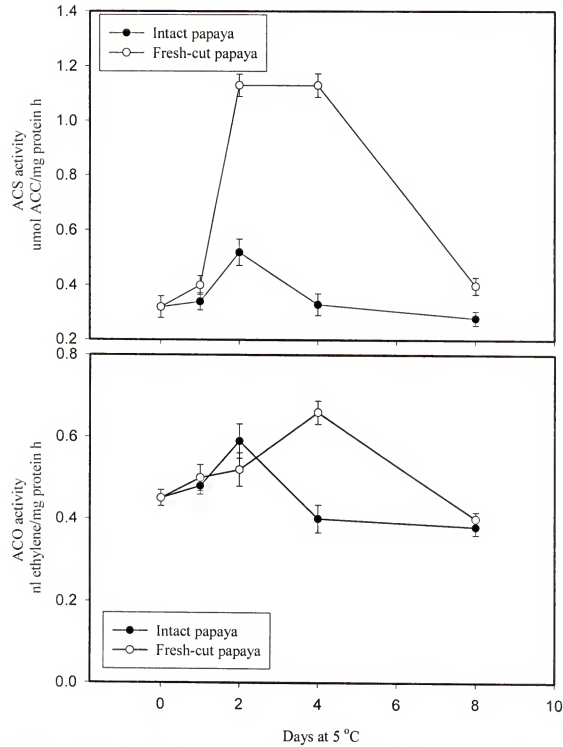

Figure 3-3. ACC synthase (A) and ACC oxidase (B) activities of intact and fresh-cut papaya fruit stored at 5 °C for 0 1, 2, 4 and 8 d. Data are the means of 6 replications. Vertical bars represent standard deviation.

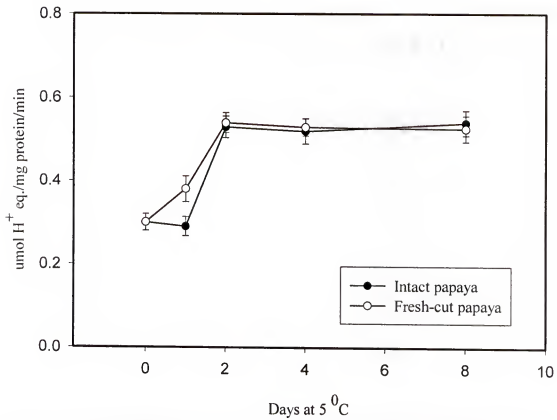

Figure 3-4. Pectinmethylesterase activity of intact and fresh-cut papaya fruit stored at 5 °C for 0 1, 2, 4 and 8 d. Data are the means of 6 replications. Vertical bars represent standard deviation.

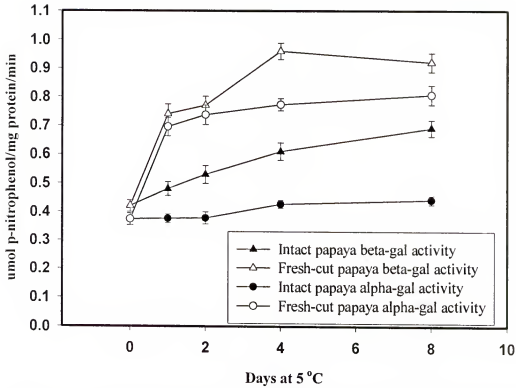

Figure 3-5. α- and β-galactosidase activities of intact and fresh-cut papaya fruit stored at 5 °C for 0 1, 2, 4 and 8 d. Data are the means of 6 replications. Vertical bars represent standard deviation.

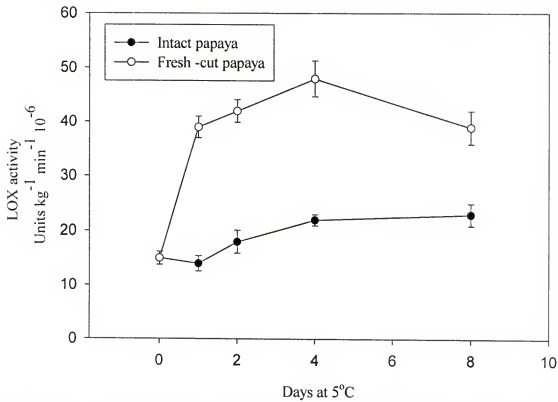

Figure 3-6. Lipoxygenase activity of intact and fresh-cut papaya fruit stored at 5 °C for 0 1, 2, 4 and 8 d. Data are the means of 6 replications. Vertical bars represent standard deviation.

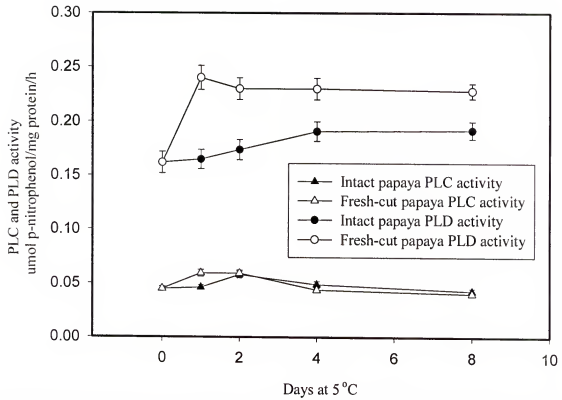

Figure 3-7. Phospholipase C and D activity of intact and fresh-cut papaya fruit stored at 5 °C for 0 1, 2, 4 and 8 d. Data are the means of 6 replications. Vertical bars represent standard deviation.

CHAPTER 4
CELL WALL-DEGRADING ENZYMES AND PECTIN SOLUBILIZATION AND DEPOLYMERIZATION IN IMMATURE AND RIPE WATERMELON FRUIT IN RESPONSE TO EXOGENOUS ETHYLENE

Introduction

Watermelon fruit have been reported to be very sensitive to ethylene. Shimokawa (1973) and Risse and Hatton (1982) demonstrated that exposure of watermelon fruit to ethylene at concentrations as low as 1 $\mu L\ L^{-1}$ brought about placental tissue deterioration and rendered the fruit unfit for consumption. Ethylene-treated fruit were soft, water-soaked, and had off-odors.

The softening of ripening fruit is primarily attributed to enzymic hydrolysis of cell wall polysaccharides (Huber 1983, Wakabayashi 2000). Ethylene plays a major role in enhancing the activity of enzymes responsible for changes occurring in cell walls during ripening. Sawamura et al. (1978) reported that exposure of tomato fruit to ethylene advanced the appearance of "pectinase" activity. Similarly, the placental tissue of watermelon fruit was macerated following exposure to ethylene, and significantly higher activity of "pectinase" activity was observed in ethylene-treated compared with air-stored fruit (Shimokawa 1973). Ethylene also hastens the appearance of Cx-cellulase activity in avocado (Tucker and Laties 1984) and polygalacturonase in tomato fruit (Biggs et al. 1986, Grierson et al. 1986).

Whereas the application of ethylene to climacteric fruits induces the accumulation of cell-wall hydrolases in parallel with the expression of other ripening processes, the response of watermelon, reportedly a nonclimacteric fruit (Elkashif et al. 1989), to

ethylene has been described as a postharvest disorder unrelated to normal ripening (Elkashif and Huber 1988a, 1988b). The accumulation of endo-polygalacturonase activity was confirmed in ethylene-treated watermelon fruit, and was accompanied by significant downshifts in polyuronide mol mass (Elkashif and Huber 1988a). Other enzymes, notably pectinmethylesterase and galactosidases/exo-galactanases have also been shown to participate in the degradation of polyuronides in ripening fruits (de Veau et al. 1993, Ranwala et al. 1992, Wakabayashi et al. 2000).

In the present study, we examined the relationship between placental-tissue water-soaking and cell wall enzyme activities and pectin depolymerization in harvested watermelon fruit treated with exogenous ethylene. Both immature and ripe fruit were examined to determine relationships between the ethylene response and fruit ripening.

Materials and Methods

Plant material

Watermelon (*Citrullus lanatus*, var. Abott and Cobb 5244, a seedless cultivar) fruit were harvested from plants at the North Florida Research and Education Center, Suwannee Valley Research Station at Live Oak, FL. Fruit were harvested at immature (firm, white flesh, fruit weight ~700 g) and commercially ripe (7-8 kg) stages. Criteria used as harvesting indices were size (immature fruit) and yellowing of the ground spot, withering of the tendril adjacent to the stem, and the sound heard when tapping the fruit with the knuckles (ripe fruit). A number of fruit were sacrificed at harvest to confirm uniformity of development. Fruit were transported to the lab on the day of harvest and sorted, washed with tap water, and rinsed with chlorinated water containing 150 $\mu L\ L^{-1}$ free chlorine.

Ethylene treatment

Fruit were placed in 125-L air-tight containers (5-6 fruit per container for both immature and ripe) at 20 °C and ventilated with the desired gas mixture. Air plus ethylene (50 $\mu L\ L^{-1}$) combinations were mixed using a flow board and administered at a flow rate of 1.2 L min^{-1} to maintain steady-state CO_2 levels below 0.05%. Relative humidity in the containers ranged from 80-90%. Fruit maintained in air were stored under the same conditions without exogenous ethylene. Ethylene levels in the treatment containers were monitored using an FID gas chromatograph (HP 5890, Palo-Alto, CA, USA) equipped with an activated alumina column.

At selected intervals during storage (0, 12 hours, 1, 3 and 6 days), fruit (6 fruit per treatment) were removed from the containers and cut longitudinally with a sterile knife. Approximately 500 grams (in pieces of approximately 9 cm^3) of the central part of the placental tissue from fruit of each treatment were frozen in liquid N_2 and stored at -30 °C for subsequent determination of polygalacturonase (PG) (EC 3.2.1.15), alpha- and beta-galactosidases (EC 3.2.1.22 and EC 3.2.1.23), and pectinmethylesterase (PME) (EC 3.2.1.11) activities, and preparation of ethanol-insoluble solids for pectin analysis.

Firmness measurements

Twenty cubes of uniform size (9 cm^3) were excised from the central part of the placental tissue and firmness of each cube was measured using an Instron Universal Testing Instrument (Model 4411, Canton, MA, USA). After establishing zero force contact with the cube, a 10-mm diameter convex probe was driven (crosshead speed 10 cm/min, 5 kg load cell) to a depth of 2 mm. The maximum force (expressed in N) recorded at 2 mm compression was used for firmness data. Firmness data were analyzed

according to a completely randomized design using GLM program of Statistical Analysis System (SAS) and Duncan's Multiple Range Test at the 5% level of significance. Ethanol-insoluble solids were prepared as described in Chapter 3.

Extraction and chromatography of water and chelator-soluble polyuronides from immature and ripe watermelon

Extraction of water-soluble polyuronides was performed by incubating ethanol-insoluble solids (20 mg) in 7 mL of deionized water for 4 h at 23 °C with stirring. The suspension was filtered through GF/C filter paper and the supernatant retained for gel chromatography. The resulting pellet was re-suspended in 7 mL of 50 mM Na-acetate, 50 mM CDTA, pH 6.5 and incubated at 23 °C with stirring for 6 h. The suspension was filtered through GF/C filter and the supernatant retained for chromatography. Uronic acids in the filtrates were determined using the procedure of Blumenkrantz and Asboe-Hansen (1973). Total uronic acids in the EIS were measured as described in Ahmed and Labavitch (1977).

Gel chromatography of polyuronides was performed as described by Chun and Huber (2000) on a bed (1.5 cm width, 27 cm length) of Sepharose CL-2B-300 packed and operated in 200 mM ammonium acetate, pH 5.0. Polyuronides (~ 0.5 mg galacturonic acid equivalents in a volume of 2.5 mL buffer) were applied to the column and eluted with the ammonium acetate buffer. Fractions of 2 mL were collected, and 0.5 mL aliquots of these used for the determination of uronic acids. Uronic acids from each treatment were chromatographed in triplicate.

The activities of polygalacturonase, pectin methyl esterase, α- and β-galactosidases were determined as described in Chapter 3.

Results

Exposure of watermelon fruit to continuous 50 μL L^{-1} ethylene resulted in a significant reduction in placental tissue firmness in both immature and ripe watermelon fruit (Fig. 4-1). Firmness declines in ethylene-treated fruit of both maturity stages were evident, within 1 day of ethylene treatment. Thereafter, placental tissue firmness showed a consistent decline, most notably between Days 3 and 6. The firmness of both immature and ripe fruits exposed to air showed no significant change during the 6-day storage period (Fig. 4-1). Consistent with the kinetics of firmness decline in ethylene-treated fruit, visible symptoms of water-soaking were evident in fruit of both maturity stages after 3 and more so after 6 days of storage (Fig. 4-2). Although the firmness of air-treated immature fruit remained significantly higher than that of air-treated ripe fruit throughout the storage period, the firmness of ethylene-treated fruit of both developmental stages converged to similar values at Day 6.

Polygalacturonase (PG) activity remained constant through the first day of storage in both immature and ripe fruit (Fig. 4-3). Thereafter, PG activity increased significantly in both immature and ripe fruits and in both air- and ethylene-treated fruits. Activity levels were significantly higher in ethylene- compared with air-treated fruit at Days 3 and 6 for immature and Day 6 for ripe fruit. After 6 days of storage, PG levels were 19% and 22% higher in ethylene-treated immature and ripe fruit, respectively, compared with air-treated controls.

The trends for total α- galactosidase (α-gal) activity varied depending on fruit maturity (Fig. 4-4A). In ripe fruit, α-gal activity decreased during the first day of storage and thereafter remained relatively constant, with higher activity evident in the ethylene- compared with air-treated fruit. In contrast, α-gal levels in both ethylene and air-treated

immature fruit showed a significant increase during the 6-day storage period, with activity reaching levels 30% higher in air- compared with ethylene-treated fruit (Fig. 4-4A). Beta-galactosidase (β-gal) activity showed a slight decrease during the first day of storage of ripe fruit. Thereafter, activities remained constant and comparable between ethylene- and air-treated fruit (Fig. 4-4B). In contrast, significant increases in β-gal activities were observed in both ethylene and air-treated immature fruit. Both the rate and magnitude of β-gal accumulation were enhanced in ethylene-treated immature fruit, with activity at 6 days of storage remaining 113% higher than that of air-treated fruit.

PME activity in immature fruit was significantly higher than levels observed for ripe fruit and was clearly influenced by ethylene treatment (Fig. 4-5). Both ethylene- and air-treated immature fruit showed declines in PME activity during the later (3 through 6 days) period of storage although activity remained significantly higher (~55%) in air compared with ethylene-treated fruit. PME activity in ethylene- and air-treated ripe fruit remained unchanged through 3 days of storage. While showing a significant increase in ethylene-treated fruit, PME activity declined slightly between days 3 and 6 in ripe fruit maintained in air.

Total uronic acid (UA) levels on per mg EIS basis were significantly higher in immature compared with ripe fruit (Fig. 4-6). In air-treated immature fruit, total UA levels increased slightly (5%) during storage whereas ethylene-treated fruit showed a significant decline. At 6 days of storage, total UA levels in ethylene-treated fruit were 10% lower than levels in air-treated fruit. A decline in total UA was noted for both ethylene- and air-treated ripe fruit, though the decline was significantly greater for the ethylene-treated (Fig. 4-6).

The influence of ethylene on the levels of water- and CDTA-soluble polyuronides is summarized in Table 4-1. In immature fruit either in air or ethylene, both CDTA- and water-soluble polyuronides increased significantly with time of storage (Table 4-1). Although ethylene effects were evident for both pectic fractions, the influence of ethylene compared with air on immature fruit was more dramatic for the CDTA-soluble fraction than for the water soluble fraction, showing 44% and 42% higher levels at Days 3 and 6, respectively. Water-soluble UA content did not change significantly in ripe fruit maintained in air. Ethylene-induced increases in water-soluble polyuronides were evident by Day 6, at which time levels were 58% higher than those from air-treated fruit. The levels of CDTA-soluble UA showed minimal change in ripe watermelon fruit during storage.

The mol mass profiles of polyuronides from watermelon fruit stored in air or 50 μL L^{-1} ethylene are illustrated in Figures 4-7 and 4-8. The mol mass distributions and downshifts differed for water- and CDTA-soluble polymers. For the water-soluble polymers, mol mass downshifts were first evident at Day 3 and day 6 for immature and ripe fruit, respectively (Fig. 4-7). Although water-soluble polyuronides were of inherently lower mol mass in ripe compared with immature fruit, ethylene-induced changes were not observed prior to 6 days of storage. As particularly evident for water-soluble polyuronides from immature fruit, mol mass downshifts were also noted for air-stored fruit although these were considerably less extensive than noted for ethylene-treated fruit.

Mol mass downshifts in CDTA-soluble polyuronides (Fig. 4-8) were first evident at Day 3 in both immature and ripe fruit. Although CDTA-soluble polyuronides from immature fruit stored in air or ethylene showed comparable mol mass downshifts

following 3 days of storage (Fig. 4-8A), the effect of ethylene was evident by Day 6, with ethylene-treated fruit showing more extensive mol mass downshifts (Fig. 4-8B). Ripe fruit showed a clear response to ethylene, with CDTA-soluble polymers exhibiting progressive mol mass downshifts first evident at day 3 (Fig. 4-8C) and becoming considerably more prominent by Day 6 (Fig. 4-8D). The mol mass distributions of CDTA-soluble polymers from air-treated ripe fruit also changed during storage but less extensively than noted for ethylene-treated fruit.

Discussion

Watermelon fruit exhibited marked softening and water-soaking in response to ethylene. Placental tissue water-soaking was visible following 3 days of ethylene exposure and acute tissue liquefaction had occurred by 6 days. These observations confirm the results of previous studies (Elkashif and Huber 1988a, Shimokawa 1973) and further show that immature fruit, in terms of the changes in placental-tissue firmness, are equally susceptible to the disorder. The ethylene-induced softening and deterioration of rind (pericarp) tissue, noted in studies of the seeded watermelon cultivar 'Charleston Gray' (Elkashif and Huber 1988a), were also observed in the present study.

The ethylene-induced decline in placental tissue firmness was associated with changes in the activity of a number of cell wall enzymes; however, none of the enzymes showed accumulation patterns in fruit of either maturity stage (immature versus ripe) that would suggest a direct role in placental tissue water-soaking. For example, ethylene-induced accumulation of α-gal and PME was restricted to immature fruit, with little or no changes in activity noted for ripe fruit. Even β-gal activity, frequently implicated in fruit softening (Carey et al. 1995, Smith and Gross 2000, Li et al. 2001), showed no change in either air- or ethylene-treated ripe fruit. Since the enzyme assays were performed using

unpurified cell-free protein extracts, the possibility remains that total activity trends masked unique changes affecting specific isoform(s). For example, the relatively constant level of total β-gal activity in ripening tomato fruit reflected declines in the activities of 2 isoforms and a 3-fold increase in activity of a pectic galactan-degrading isoform (Pressey 1983).

The only consistent response among the cell wall enzymes to ethylene treatment in both immature and ripe fruit was increased PG activity. Earlier reports (Elkashif and Huber 1988a, Shimokawa 1973) also implicated an involvement of PG in the water-soaking syndrome. In studies of ethylene treatment of 'Charleston Gray' watermelon fruit, basal levels of PG were negligible and showed no increase in air-treated fruit, even for extended storage periods (Elkashif and Huber 1988a). Furthermore, symptoms of water-soaking did not develop in air-stored 'Charleston Gray.' In contrast to the seeded 'Charleston Gray, the seedless Abott and Cobb 5244 showed measurable levels of PG throughout storage (Fig. 4-3), even in air-treated fruit that showed little change in placental tissue firmness.

Although differences in extractable PG activity in air- and ethylene-treated watermelon fruit within each maturity class were not dramatic, the decline in placental tissue firmness was temporally correlated with significant mol mass downshifts in water- and CDTA-soluble polyuronides. The downshifts in mol mass were extensive, particularly in ripe fruit, with both water- and CDTA-soluble polymers fractionating near the inclusion limit of the column (Fig. 4-7D, 4-8D). The differences in polyuronide depolymerization between air- and ethylene-treated fruit were possibly greater than was apparent from the gel filtration data. The significant decline in total UA content in

response to ethylene treatment (Fig. 4-6) could reflect depolymerization to products (oligouronides) not quantitatively recovered in alcohol-insoluble powders (Dumville and Fry 2000). In this regard, the depolymerization of polyuronides, as assessed from gel filtration analyses, in ethylene-treated watermelon fruit is similar in magnitude to that observed for avocado, which also shows a significant decline in total UA levels during ripening (Wakabayashi and Huber 2001). In contrast, the more limited hydrolysis of polyuronides in ripening tomato fruit is not accompanied by changes in the total UA content (Huber and Lee 1986). It is evident that the acute water-soaking in ethylene-treated watermelon fruit can not be explained solely on the basis of polyuronide degradation. The unusually extensive polyuronide depolymerization occurring during avocado ripening (Huber and O'Donoghue 1993, Wakabayashi et al. 2000) is not accompanied by the symptoms of water-soaking and liquefaction observed in watermelon fruit.

Changes in enzyme activities (PG, PME, α- and β-gal) induced by ethylene exposure were typically paralleled by similar though less marked changes occurring in air-treated fruit. This observation indicates that enzyme induction does not require ethylene or that endogenous levels of the gas are sufficient to induce ethylene responses. While we did not measure fruit ethylene synthesis in the present experiments, ethylene production in air-stored 'Charleston Gray' watermelon fruit did not exceed 0.1 $\mu L\ kg^{-1}\ h^{-1}$ and the fruit did not exhibit water-soaking, even following extended periods of air storage (Elkashif et al. 1988a).

Chatenet et al. (2000) concluded that water-soaking affecting the mesocarp of Charentais cantaloupe melons during the late stages of ripening was not caused by

ethylene. The authors reported increased water mobility, the presence of large intercellular spaces, a significant depletion of wall calcium, and no change in the expression of genes encoding ACC synthase and oxidase. It is evident, however, that ethylene contributes directly to the water-soaking phenomenon in watermelon fruit. Both mRNA levels and activities of ACC synthase and oxidase increase significantly in watermelon fruit in response to ethylene (Chapter 4). As more direct evidence for the causative role of ethylene, watermelon fruit pre-treated with the ethylene-action inhibitor, 1-methylcyclopropene (1-MCP), showed no symptoms of water-soaking following an 8-day exposure to 50 $\mu L\ L^{-1}$ ethylene (Mao and Huber unpublished). In contrast, 1-MCP did not prevent water-soaking in cantaloupe melons (Chatenet et al. 2000). It seems evident that the water-soaking phenomena observed in watermelon and cantaloupe melons occur via different mechanisms.

An explanation for the extensive degradation of polyuronides in ethylene-treated watermelon in spite of minimal differences in the activities of cell wall hydrolases may involve ethylene-induced membrane permeability changes. Elkashif and Huber (1988b) reported that enhanced electrolyte leakage was an early response in the placental tissue of watermelon fruit exposed to ethylene. A relationship between ethylene and increased membrane permeability has been observed for leaf (Bailey et al. 1990), floral (Suttle and Kende 1980, Celikel and van Doorn 1995) and fruit (Lacan and Baccou 1996) tissues. Altered permeability could result in an efflux of solutes including organic acids into the apoplast, altering the binding of wall-associated Ca^{+2} (Ferguson et al. 1980) and possibly also resulting in altered apoplastic pH (Almeida and Huber 1999). Both events could selectively enhance or suppress the activity of pre-existing cell wall hydrolases,

independent of changes in enzyme quantities. The depletion of cell-wall Ca^{+2} (Chatenet et al. 2000) could itself increase membrane degradation through induction of lipases as a result of movement of Ca^{+2} to the cytosol. In support of this hypothesis, we have found significant increases in the activities of lipoxygenase and phospholipases C and D in ethylene- compared with air-treated ripe watermelon fruit (Chapter 5).

Table 4-1. Changes in CDTA and water-soluble polyuronides of ripe and immature watermelon fruit in response to ethylene treatment (50 μL L^{-1}) during storage at 20 °C for 0 and 12 hours and 1,3 and 6 days.

	Water-sol. polyuronides (μg/mg EIS)				CDTA-sol. polyuronides (μg/mg EIS)			
	Ripe fruit		Immature fruit		Ripe fruit		Immature fruit	
Days	Air	Ethylene	Air	Ethylene	Air	Ethylene	Air	Ethylene
0	23.1a	-----	42.4b	-----	66.8a	-----	24.7b	-----
3	24.5a	27.4b	45.6b	48.9b	68.9a	72.2b	25.4b	36.1b
6	26.2a	41.3a	61.2a	69.5a	69.8a	75.8a	33.1a	47.8a

Data are the means of 6 replications. Means within each column followed by the same letter are not significantly different at the 5% level of significance.

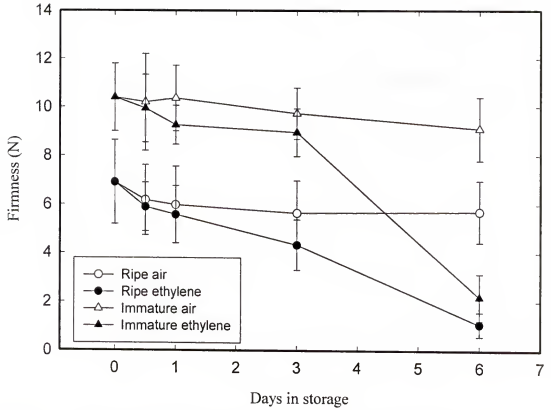

Figure 4-1. Firmness of immature and ripe watermelon fruit exposed to air or ethylene (50 uL L^{-1}) at 20 °C for 0 and 12 hours and 1,3 and 6 days. Data are the means of 20 replications. Vertical bars represent standard deviation.

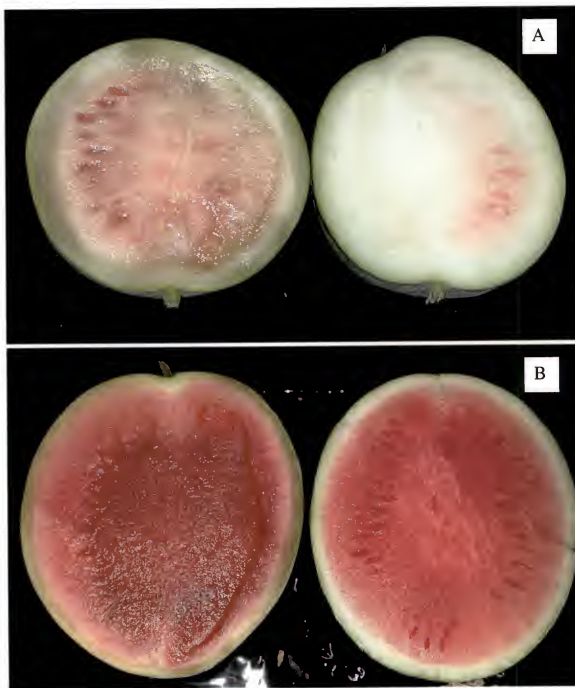

Figure 4-2. Ethylene-induced water-soaking in immature (A) and ripe (B) fruits.

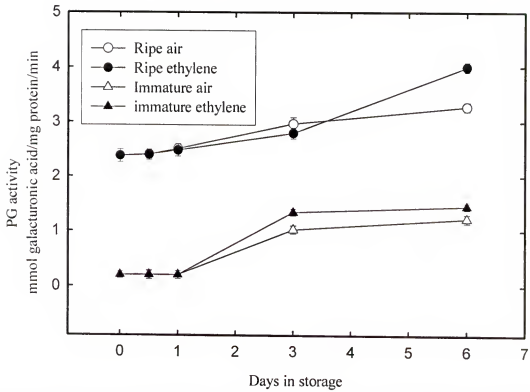

Figure 4-3. Polygalacturonase activity of immature and ripe watermelon fruit exposed to air or ethylene (50 uLL^{-1}) at 20 °C for 0 and 12 hours and 1,3 and 6 days. Data are the means of 6 replications. Vertical bars represent standard deviation.

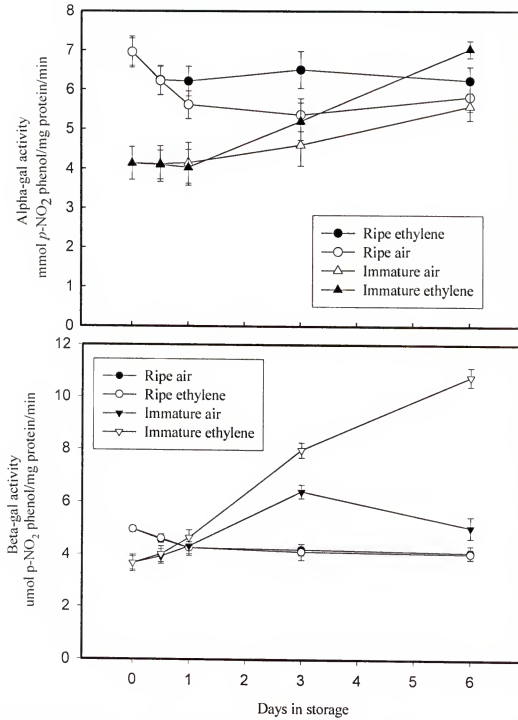

Figure 4-4. Alpha- and Beta-galactosidase activities of immature and ripe watermelon fruit exposed to air or ethylene (50 uLL^{-1}) at 20 °C for 0 and 12 hours and 1,3 and 6 days. Data are the means of 6 replications. Vertical bars represent standard deviation.

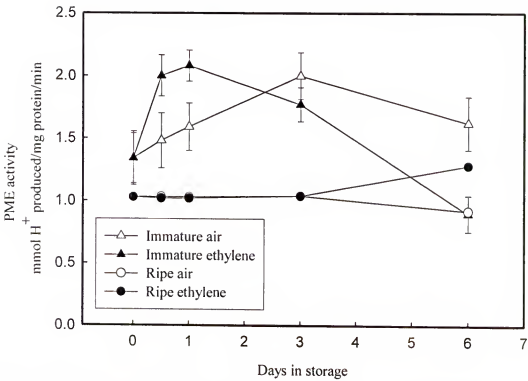

Figure 4-5. Pectin methylesterase activity of immature and ripe watermelon fruit exposed to air or ethylene (50 uLL^{-1}) at 20 °C for 0 and 12 hours and 1,3 and 6 days. Data are the means of 6 replications. Vertical bars represent standard deviation.

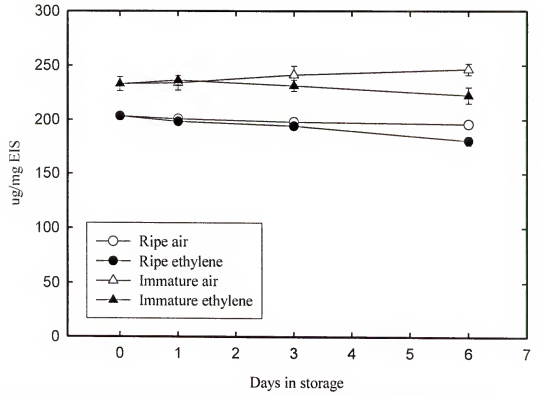

Figure 4-6. Total polyuronides content of immature and ripe watermelon fruit exposed to air or ethylene (50 uLL^{-1}) at 20 °C for 0 and 12 hours and 1,3 and 6 days. Data are the means of 6 replications. Vertical bars represent standard deviation.

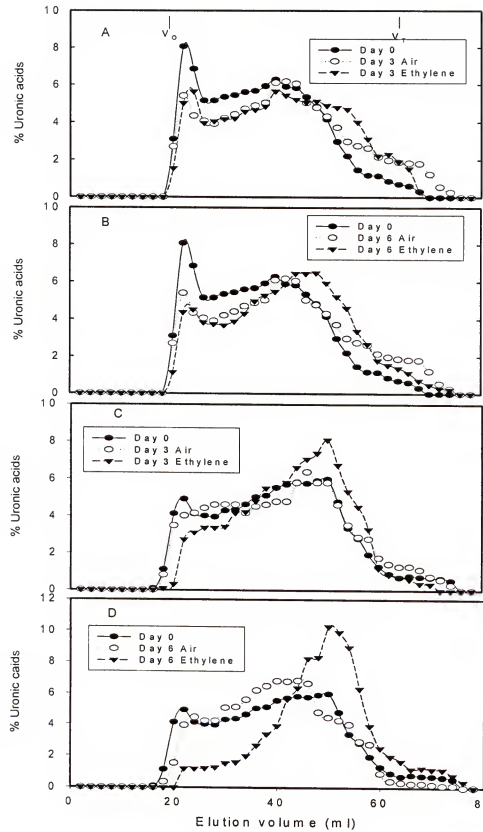
A
B
C
D
V_o
V_t
Day 0
Day 3 Air
Day 3 Ethylene
Day 6 Air
Day 6 Ethylene
% Uronic acids
% Uronic acids
% Uronic acids
% Uronic caids
Elution volume (ml)

Figure 4-7. Mol mass profiles of CDTA-soluble polyuronides from immature (A,B) and ripe (C,D) watermelon fruit exposed to air or ethylene (50 ul l^{-1}) at 20 °C for 0, 3 and 6 days. Polyuronides (0.5 mg galacturonic acid equivalents) were applied to a CL-2B-300 (1.5 x 27 cm) column operated with a mobile phase of 200 mM ammonia acetate, pH 5.0. Fractions of 2 ml were analyzed for uronic acids.

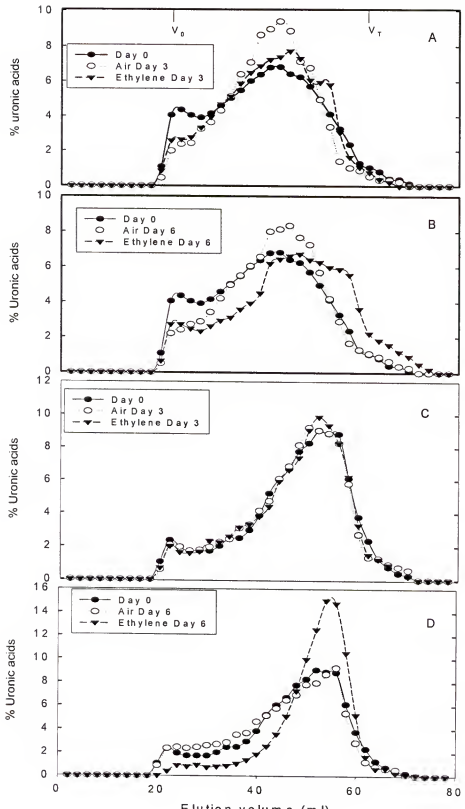
A
Day 0
Air Day 3
Ethylene Day 3
V_0
V_T
% uronic acids
B
Day 0
Air Day 6
Ethylene Day 6
% Uronic acids
C
Day 0
Air Day 3
Ethylene Day 3
% Uronic acids
D
Day 0
Air Day 6
Ethylene Day 6
% Uronic acids
Elution volume (ml)

Figure 4-8. Mol mass profiles of water-soluble polyuronides from immature (A,B) and ripe (C,D) watermelon fruit exposed to air or ethylene (50 ul l^{-1}) at 20 ^{o}C for 0, 3 and 6 days. Polyuronides (0.5 mg galacturonic acid equivalents) were applied to a CL-2B-300 column (1.5 x 27 cm) operated with a mobile phase of 200 mM ammonia acetate, pH 5.0. Fractions of 2 ml were analyzed for uronic acids.

CHAPTER 5
REGULATION OF GENE EXPRESSION BY EXOGENOUS ETHYLENE IN WATERMELON FRUIT

Introduction

Watermelon (*Citrullus lanatus*) fruit are very sensitive to exogenous ethylene, exhibiting extensive placental and pericarp softening and water soaking following short exposure to the gas (Elkashif and Huber 1988a, Chapter 4). The water soaking phenomenon is accompanied by enhanced electrolyte efflux (Elkashif and Huber 1988b), and both xyloglucan and pectin depolymerization (Huber et al. 2002). Ethylene has been shown to be involved in increasing the activity of several cell wall enzymes in ripening fruit including polygalacturonase (EC 3.2.1.15) in tomato (Grierson et al. 1986) and endo-β-1,4-glucanase (EC 3.2.1.4, C_x-cellulase) activity in avocado fruits (Awad and Yang 1979, Tucker and Laties 1984). Ethylene has been shown to induce the expression of many genes including ripening related genes, ethylene biosynthetic genes, the genes of ethylene signal transduction pathway, and cell wall enzymes including polygalacturonase and C_x-cellulase, (reviewed in Jiang and Fu 2000, and Bruxelles and Roberts 2001, Hoeberichts et al. 2002). The role of ethylene in induction of these genes has been confirmed by application of chemical inhibitors of ethylene perception (Sisler and Blakenship 1993, Sisler and Serek 1997) or in transgenic plants with altered ethylene production levels (Klee et al. 1990, Oeller et al. 1991). For example, Davies et al. (1988) showed that treating breaker tomato fruit with silver ions can prevent the appearance of ripening-related mRNAs and proteins. Hoeberichts et al. (2002) reported that 1-MCP

treatment reduced the accumulation of mRNA of phytoene synthase 1, expansin 1, and 1-amonocyclopropane-1-carboxylic acid oxidase 1 in tomato. Watermelon fruit also exhibit enhanced PG activity in response to exogenous ethylene (Elkashif et al. 1988a). Both immature and fully ripe fruit exhibit similar water soaking in response to ethylene exposure (Chapter 4), suggesting that the phenomenon might represent a disorder unrelated to normal ripening. The water stress-induced accumulation in immature cucumber fruit of a transcript for PG, and inhibition of this response by the ethylene antagonist 1-methylcyclopropene (Kubo et al. 2000) argue that the accumulation of this enzyme represents an ethylene-mediated stress response in some members of the *Cucurbitaceae*. The injury-induced breakdown of endocarp tissue in immature cucumber fruit (Abbott et al. 1991) supports this idea. Reports of both nonclimacteric (Elkashif et al. 1989) and climacteric (Mizuno and Pratt 1973) watermelon cultivars would suggest for at least climacteric types that water soaking should be expressed even in the absence of exogenous ethylene. The development of water soaking and changes in the trends of several cell wall hydrolases were in fact noted in air-stored seedless watermelon Abott and Cobb and 5244, but the appearance of water soaking was significantly delayed compared with fruit exposed to ethylene (Chapter 4). Long-term (months) air-storage of the seeded 'Charleston Gray' watermelon, that exhibits nonclimacteric behavior (Elkashif et al. 1989), was accompanied by the development of placenta mealiness rather than tissue liquefaction characteristic of ethylene exposure (Elkashif and Huber 1988a).

The objective of the present study was to address the temporal relationship between placental-tissue water-soaking and transcript levels in harvested, commercially ripe and immature watermelon fruit exposed to ethylene. Emphasis was on mRNA abundance for

several cell wall and membrane hydrolases, and ethylene biosynthetic enzymes. The activities of these enzymes have been shown for both immature and ripe watermelon fruit to respond to ethylene exposure (Chapter 4).

Materials and Methods

Plant material

Watermelon (*Citrullus lanatus*, var. Abott and Cobb 5244, a seedless variety) fruit were grown using standard commercial practices at the University of Florida Suwannee Valley Research Station at Live Oak, FL. Fruit were harvested at immature (firm, white flesh, fruit weight ~700 g) and commercially ripe (firm, red flesh, 7-8 kg) stages. Harvesting indices included size and yellowing of the ground spot, withering of the tendril adjacent to the stem, and the sound heard when tapping the fruit with the knuckles (ripe fruit). Fruit were transported to the lab on the day of harvest and sorted, washed with tap water, and rinsed with chlorinated water containing 150 $\mu L\ L^{-1}$ free chlorine.

Ethylene treatment was performed as described in Chapter 4. At selected intervals during storage (0, 12 h, 1, 3 and 6 days), fruit (6 fruit per treatment) were removed from the containers and cut longitudinally. Approximately 100 grams (in pieces of approximately 9 cm^3) of the central portion of the placental tissue freed of undeveloped or aborted seed structures were frozen in liquid N_2 and stored at -80 ^0C for subsequent RNA extraction and determination of the specific activities of ACC synthase and ACC oxidase, lipoxygenase, and phospholipases C and D.

RNA Extraction

Total RNA was isolated from air and ethylene-treated fruits using the protocol of Strommer et al. (1993). Briefly, 20 g of placental tissue were ground to a fine powder in liquid N_2 and freeze-dried. One g of the dried tissue was added to 10 mL of extraction

buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % sarcosyl and 0.1 M β-mercaptoethanol) stirring on a stir plate and then 1 mL of 2 M NaOAc, pH 4 was added and the suspension was vortexed for 30 s. After addition of 10 mL of water-saturated phenol, the mixture was vortexed and 6 mL of chloroform:isoamyl alcohol (24:1, v/v) were added. After vortexing for 30 s, the suspension was centrifuged at room temperature (5,000 x g) for 10 min. The aqueous phase was transferred to a clean centrifuge tube and an equal volume of chloroform:isoamyl alcohol was added and centrifuged at room temperature (5,000 x g) for 10 min. After transferring the aqueous phase to a clean centrifuge tube, an equal volume of isopropanol was added, stirred, and incubated overnight at -20 °C. After centrifuging at 10,000 x g (4 °C) for 30 min the nucleic acid pellet was resuspended in 500 µL RNAse free water. RNA was precipitated via addition of 500 µL 4 M LiCl and incubation at 0 °C for 3 h. After centrifugation at top speed (Heraeus, 15,000 rpm, room temperature) for 5 min, the pellet was washed twice with 70% ethanol and then air-dried and resuspended in 200 µL of diethylpyrocarbonate-treated, autoclaved water. The total RNA was used for Northern blot analysis as described below.

Northern Blot Analysis

Total RNA (10 µg) from both air- and ethylene-treated fruit was fractionated on a 1.5% agarose-formaldehyde gel (1.5% agarose, 20 mM MOPS, 5 mM sodium acetate (pH 7.0), 1 mM EDTA, 1% formaldehyde, 0.1 µg/mL ethidium bromide) and then transferred onto a nylon membrane (Roche, Indianapolis, IN, USA) by capillary transfer in 10X SSC buffer (1.5 M NaCl, 0.3 M Sodium citrate, pH 7.0). After 12 h transfer time, the membrane was rinsed briefly in 2X SSC to remove excess salt, and placed in a (Bioslink) UV cross-linker (312 nm). Blots were pre-hybridized in DIG-Easy Hyb

solution and then hybridized with the DIG-labeled probe according to manufacturers instructions (Roche). Following hybridization, washing and detection were carried out as described (Roche). Equivalence of RNA loadings from one sample to another was checked by examination of the gel over a UV transilluminator (Fisher Scientific, USA). The signals were quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The expression levels of each transcript in the control fruit (Day 0) were set to an arbitrary value of 1.0, to which all other time points for both air- and ethylene-treated fruit were normalized to generate an induction value (fold induction).

Probes for ACC synthase and polygalacturonase were kindly provided by Dr. TG McCollum (US Horticultural Research Laboratory, Fort Pierce, Fl). Expansin (*At-EXP1*), originally from *Arabidopsis thaliana* (Scherban et al. 1995) and beta-galactosidase (*TBG4*) from tomato (Smith et al. 1998) probes were gifts of Dr. DJ Cosgrove (Pennsylvania State University, University Park, PA) and Dr. KC Gross (Horticultural Crops Quality Laboratory, Belstville, MD), respectively. Watermelon pectin methyl esterase (PME), ACC oxidase (ACO), lipoxygenase (LOX), phospholipase C (PLC) and D (PLD) probes were prepared by polymerase chain reaction (PCR) amplification using reverse transcribed RNA as template and the following combinations of primers:

for PME (1885-bp fragment; accession number AF152172), 5'-TCGCTGAAATGTTGGATTCC-3' and 5'-TTGTTATTTTGCCGTCAGCC-3'

for ACO (890-bp fragment; accession number AY077461), 5'-TCATCAACATGGAGGGTCTCA-3' and 5'-TTCATGGCATCAAATCTC-3'

for LOX (2786-bp fragment; accession number X92890), 5'-CATTGAAGGGGCCTTGAATA-3' and 5'-TTCCAAATCCAACAGAGCCT-3'

for PLC (1620-bp fragment; accession number Y15253), 5'-TTTAAGCTCGGCATATCGGA-3' and 5'-TGTTTGCCCACCAAAGTCA-3'

for PLD (2677-bp fragment; accession number D73410), 5'-TCATCAATCACGGTGACCTTG-3' and 5'-ATAACGTCGAATCCCTCT-3'

The PCR products were cloned (TA Cloning system, Invitrogen), sequenced (ICBR, University of Florida, Gainesville), and compared to the sequences of the corresponding genes available in the NCBI database (BLASTN and BLASTX programs with default settings, Altschul et al. 1997) to verify sequence homology. The probes were labeled with Digoxygenin dUTP using DIG High Prime labeling kit following manufacturers instructions (Roche).

The activities of ACC synthase and oxidase, lipoxygenase, phospholipases C and D were determined as described in Chapter 3.

Results

Previous studies of Abott and Cobb 5244 seedless watermelon fruit demonstrated that immature and fully ripe fruit develop water soaking in response to ethylene exposure (Chapter 4). The ethylene response is initiated within 12 h of application, and is evident as enhanced placental tissue softening, followed after 3 d of exposure by a rapid decline in firmness to values representing about 86% (immature) and 81% (ripe) of values for fruit at the start of the experiment. The development of water-soaking was accompanied by changes in the activity of PG, α- and β-gal activities, and PME activity. A clear involvement of these enzymes in water soaking was not apparent, however, since other than for PG, the activity trends of the cell wall enzymes were either unaffected by ethylene or showed very different patterns of activity in immature compared with ripe fruit. In this study, the relationship between water soaking and the expression patterns of

enzymes targeting cell walls and membranes and ethylene biosynthetic enzymes was investigated. Under the conditions for hybridization employed, all of the probes examined would be predicted to hybridize only with transcripts of high sequence similarity but it is possible that some cross-hybridization to mRNAs of closely related gene family members also occurs. In all analyses, transcript abundance data were expressed as relative values with respect to the levels for the Day 0 control fruit. A two-fold change in transcript abundance as compared to levels at Day 0 was considered as significant.

As shown in Figure 5-1, polygalacturonase (PG) mRNAs accumulated in both air- and ethylene-treated immature and ripe watermelon fruit; however, for fruit of both developmental classes, the induction level for PG mRNA was higher in fruit exposed to ethylene at all time periods during storage. PG transcript levels in air- and ethylene-treated immature watermelon increased to maximum factors of 2.5 and 3.3 fold respectively, after 3 d exposure to ethylene, thereafter decreasing slightly (Fig. 5-1A). In air-treated ripe fruit, PG mRNA abundance increased a total of 1.9 fold through 6 d of storage whereas induction values of 2.8 were noted for ethylene-treated ripe fruit after only 3 d, declining thereafter to induction values of about 2.4 at Day 6 (Fig. 5-1B).

In immature fruit, PME mRNA increased approximately 1.3 fold within 12 h in air-treated fruit, and decreased significantly during the remainder of storage (Fig. 5-2A). On the other hand, PME transcript levels in ethylene-treated immature fruit increased ~ 1.4 fold within 24 h and then decreased toward 6 d of storage. In ripe fruit, the levels of mRNAs encoding PME increased 3.1- and 1.8 fold at 3 d of storage in ethylene- and air-treated ripe fruit, respectively (Fig. 5-2A).

The change in mRNA levels encoding ß-galactosidase (ß-Gal) depended on the ripening stage of the fruit (Fig. 5-3). In immature fruit ß-Gal transcripts accumulated in both air and ethylene treated fruit attaining max levels (3.4 fold in air-treated fruit and 5.4 fold in ethylene-treated fruit) after 1 d in fruit maintained in air and 6 d in ethylene-treated fruit during storage (Fig. 5-3A). However, in ripe watermelon fruit, ß-gal transcript levels decreased significantly in both air- and ethylene-treated fruits during the storage period (Fig. 5-3B).

While showing ~ 1.4 fold elevation after 6 d in air-treated fruit, the levels of expansin (Exp) transcripts in immature fruit increased more than 2 fold only after 24 h of ethylene exposure and then declined to about 1.4 fold above the uninduced levels at day 0 (Fig. 5-4A). Similar patterns of expression were observed for ripe watermelon fruit during the storage period. Exp mRNA in ethylene-treated ripe fruit increased ~ 3 fold after 24 h of ethylene exposure and then declined to the levels below the basal level at Day 0 after 6 d of storage (Fig. 5-4B). In air-treated ripe fruit Exp gene expressed slightly (1.4 fold) after 6 d.

Immature fruit seemed to accumulate ACC oxidase (ACO) transcripts in an ethylene independent fashion. ACO transcripts induced ~ 4 fold after 3 d of storage in ethylene-treated fruit, but air-treated fruit showed a gradual increase during storage with max induction of about 2.7 fold by Day 6 (Fig. 5-5A). ACO activity showed similar trends with transcript accumulation. The ACO activity of immature fruit treated with ethylene showed an increase (200% compared to Day 0) during storage with activity reaching to significantly higher levels (50%) in ethylene-treated fruit compared with the activity in fruit maintained in air after 6 d (Fig. 5-5C). A parallel but delayed increase in

ACO activity (> 100% increase) was also noted in air-treated fruit confirming the increase in transcript levels of ACO. The ACO mRNA levels in ethylene-treated ripe fruit elevated about 4.8 fold after 3 d and thereafter declined to about 4 fold above the levels at Day 0 (Fig. 5-5B). ACO activity in ethylene-treated ripe fruit increased significantly (61%) within 12 h of ethylene exposure and continued to increase up to day 6 reaching to a max level at Day 6 at which time the activity was 224% higher than the activity level in air-treated fruit (Fig. 5-5C). Air-treated ripe fruit showed no change in ACO activity up to 3 d and then an increase of ~ 35% relative to the activity level at Day 0. The increase in the activity of air-treated fruit corresponds to the significant increase in ACO mRNA accumulation after 6 d.

The levels of ACC synthase (ACS) transcripts and activity changed differentially in response to exogenous ethylene and fruit maturity (Fig. 5-6). ACS mRNA levels showed significant increases in both air- and ethylene-treated immature fruit during storage, although transcript levels in ethylene-treated fruit remained relatively higher than in air-treated fruit throughout storage (Fig. 5-6A). ACS transcripts accumulated more than 2.5 fold within 12 h in both air- and ethylene-treated fruit. However, ACS mRNA levels continue to increase in ethylene-treated fruit up to 24 h. ACS activity in ethylene-treated fruit showed a significant increase after 3 d of storage and continue to increase during the rest of the storage period reaching levels 156% higher than the activity level at Day 0 (Fig. 5-6C). On the other hand, ACS activity in air-treated fruit increased significantly only after 24 h showing approximately 89% increase following 6 d of storage. Although ACS activity was enhanced in both air-and ethylene-treated fruit, the latter showed 35% higher activity than the former after 6 d confirming higher transcript levels detected in

ethylene treated fruit. ACS transcripts decreased slightly in air-treated ripe watermelon fruit during storage. However, exogenous ethylene induced transcript accumulation more than 3.5 fold within 12 h of exposure in ripe fruit, and ACS transcript levels remained significantly higher than the levels in air-treated fruit during storage (Fig. 5-6B). ACS activity followed similar trends to the transcript levels in both air- and ethylene-treated fruit. While remaining relatively unchanged during storage in air-treated fruit, ACS activity enhanced markedly (over 530% compared to Day 0) in ethylene treated fruit (Fig. 5-6C).

Several enzymes targeting membrane lipids were also analyzed. Northern blot analysis with a lipoxygenase (LOX) probe revealed that in immature fruit maintained in air, LOX transcripts decreased 2.5 fold after 24 h and then remained constant towards the end of the storage (Fig. 5-7A). Exogenous ethylene induced LOX levels by 2.8 fold only after 24 h, reaching to a max at Day 3. LOX activity in immature fruit maintained in air as is the case for LOX transcripts, decreased slightly during storage, while the activity in fruit treated with ethylene increased approximately 62% after 6 d (Fig. 5-7C). LOX mRNAs accumulated 2.5 fold in ethylene-treated ripe watermelon fruit following 3 d and further increasing 3 fold by Day 6, while transcript levels in air-treated fruits remained unchanged after 6 d (Fig. 5-7B). Similarly LOX activity in fruit maintained in air remained relatively constant during storage (Fig. 5-7C). However, LOX activity in parallel to the LOX mRNA accumulation enhanced significantly after 6 d in ethylene-treated fruit.

Phospholipase C (PLC) mRNA levels in immature fruit maintained in air showed a transient decrease during storage while PLC transcripts accumulated more than 2 fold

within only 12 h of ethylene exposure and remained significantly higher than the levels in air-treated fruit throughout storage (Fig. 5-8A). PLC mRNA in air-treated ripe fruit declined significantly after 24 h and thereafter increased to slightly above the basal level at Day 0 (1.3 fold), while PLC transcripts in ethylene-treated fruit increased ~2.4 fold after 24 h and then decreased slightly through Day 6 of storage (Fig. 5-8B). PLC activity increased significantly in ethylene-treated ripe and immature fruit within 24 h of storage (41% and 69% respectively) and continued to increase during the rest of the storage period (Fig. 5-8C). After 6 d of storage PLC activity increased 137% and 236% in ethylene treated ripe and immature fruits, respectively, compared to the levels at Day 0.

Phospholipase D (PLD) transcript levels in immature fruit elevated by 3 and 4 fold in air- and ethylene-treated fruit respectively within 12 h and thereafter showing a reduction in both air- and ethylene-treated fruit (Fig. 5-9A). However, PLD mRNA level in ethylene-treated fruit remained higher than the level in air-treated fruit throughout the storage. PLD activity in immature fruit maintained in air increased 174% following 3 d and then declined towards 6 d (Fig. 5-9C). PLD activity in ethylene-treated fruit enhanced 516% after 6 d of storage and remained relatively higher than that of fruit treated with air. By Day 6, PLD activity of ethylene-treated fruit was 248% higher than that of air-treated fruit. PLD mRNA levels showed a reduction of 4 fold in fruit maintained in air after 6 d, but the transcript accumulation in fruit treated with ethylene enhanced approximately 2 fold within 12 h and reached a max level after 3 d of storage and thereafter declined to the level 1.3 fold above the basal level at Day 0 (Fig. 5-9B). PLD activity of ethylene and air treated ripe fruit increased 23 and 68% respectively within 24 h. While remaining relatively unchanged during the rest of the storage period in

air treated fruit, PLD activity in ethylene treated fruit continued to increase reaching a max at Day 6 at which time the activity of ethylene-treated fruit was 122% higher than that of air-treated fruit (Fig. 5-9C).

Discussion

Exposure of watermelon fruit to exogenous ethylene brings about rapid tissue softening and watersoaking within a short period of exposure (Chapter 4). Extensive pectin depolymerization and solubility in ethylene treated fruit, with an enhanced polygalacturonase activity has also been reported. Northern analysis on ripe and immature watermelon fruit revealed that PG and EXP mRNA levels increased in response to ethylene exposure in both ripe and immature watermelon fruit suggesting a possible involvement of these two cell-wall enzymes in the watersoaking phenomena. The accumulation of β-gal and PME transcripts, however, were limited to immature and ripe fruit respectively. The lack of an induction of β-gal in ripe fruit and PME in immature fruit in response to ethylene suggest that β -gal and PME possibly are not influential in ethylene-induced watersoaking in watermelon fruit. Consistent with the transcript data, no association between the development of watersoaking and PME and α- and β-gal specific activity in watermelon fruit has been determined (Chapter 4).

ACO and ACS transcripts increased significantly in both ripe and immature fruit in response to ethylene exposure, and were accompanied by enhanced activity of both ethylene biosynthetic enzymes possibly resulting in elevated ethylene biosynthesis. Increased ethylene synthesis in response to exogenous ethylene has been reported in many fruits including melon (Shiomi et al. 1999). Although 2-5 fold increases in transcript levels were observed for both ethylene biosynthetic enzymes, increases in their activities was approximately 5-10 fold suggesting an enhancement of the activity together

with increased protein synthesis. The positive correlation observed between the activities of both ethylene biosynthetic enzymes and their transcript levels suggests that the ACO and ACS activities in watermelon fruit is regulated, at least in part at the transcriptional level (Kende 1993).

Exposure of various plant tissues to ethylene has been reported to also cause an increase in membrane permeability, an indication of membrane disintegration (Ferguson et al. 1980, Elkashif and Huber 1988a). The analysis of enzymes targeting membrane lipids revealed significant inductions in the levels of both activity and transcripts of LOX, PLC and PLD. Although remained relatively constant or decreased in air-treated fruit of ripe and immature watermelon fruit respectively, LOX mRNAs accumulated significantly in response to exogenous ethylene during the storage indicating a possible involvement of LOX-dependent lipid hydrolysis to the water-soaking syndrome. LOX is known to be involved in the production of defense-related signaling molecules (Creelman et al. 1992, Albrecht et al. 1993, Laudert et al. 1996) or participation in autocatalytic peroxidation reactions (Hildebrand 1989). LOX hydroperoxides can contribute to tissue damage through inactivation of protein synthesis and deterioration of cellular membranes. Similar to transcript levels, LOX activity increased markedly during storage in response to ethylene in both ripe and immature watermelon fruit coinciding with the increase in transcript level thus further supporting the role of LOXs in water-soaking disorder in watermelon fruit. Accumulation of PLD and PLC transcripts was an early response to ethylene, the transcript levels of which increased two and four fold respectively in immature fruit within 12 h accompanied by an increase in the activities of both lipases. Similarly a significant but delayed increase in mRNAs and activities of both lipases were

observed in ripe fruit indicating a possible membrane lipid degradation in response to exogenous ethylene. Consistent with this interpretation, an increase in electrolyte leakage in response to exogenous ethylene has been reported by Elkashif and Huber (1988a) in ethylene treated 'Charleston Gray' watermelon fruit. Generally, the activity of lipolytic enzymes including phospholipases and LOXs increases during senescence (Todd et al. 1990, Wang 2001), the former activities resulting in the release of membrane unsaturated fatty acids that can serve as substrates for LOX. PLC releases inosidine triphosphate that promotes oscillations and increases in cytoplasmic Ca^{2+} (Staxen et al. 1999). The increase in Ca^{2+} may enhance PLD association with membranes, resulting in PLD activation (Wang 2000, Zheng et al. 2000). The activation of PLD may generate phosphatidic acid, stimulating other lipolytic activities such as PLA_2 as shown in mammalian systems (Liscovitch et al. 2000). LOX-induced free radials may also directly act on cell-wall polysaccharides further contributing to the softening observed in watersoaked fruit. Dumville and Fry (2000) and Schweikert et al. (2000) have suggested a role for radical-based mechanisms in polysaccharide breakdown in plant development, providing a possible relationship between peroxidative lipid metabolism and tissue softening. The absence of significant increases in both mRNA and activities of LOX, PLD and PLC enzymes in air-treated fruit suggests that these lipases are induced by exogenously applied ethylene possibly resulting in membrane lipid hydrolysis and leading to the leakage of the cytoplasmic fluid into the apoplast, thus generating watersoaking phenomena and the resulting change in apoplastic conditions may enhance cell wall hydrolase activity leading to extensive cell wall polysaccharide degradation.

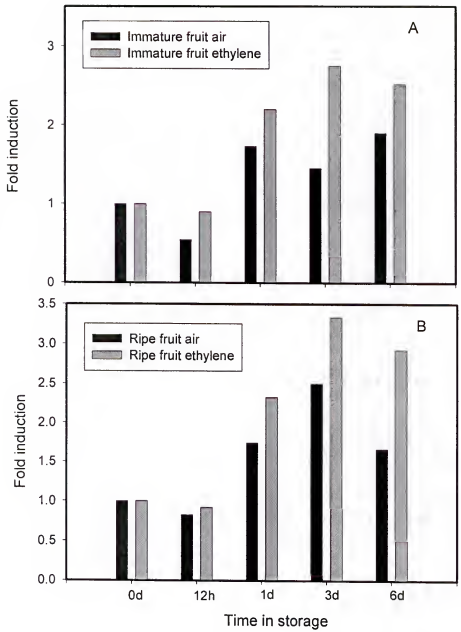

Figure 5-1. Northern blot analysis with a watermelon *PGII* probe of total RNA (10 μg) from air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3, and 6 days. Immature fruit (A), ripe fruit (B).

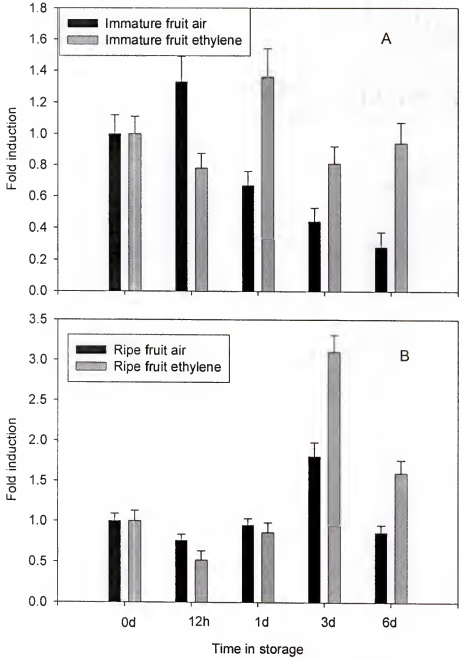

Figure 5-2. Northern blot analysis with a watermelon PME probe of total RNA (10 µg) from air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3, and 6 days. Immature fruit (A), ripe fruit (B).

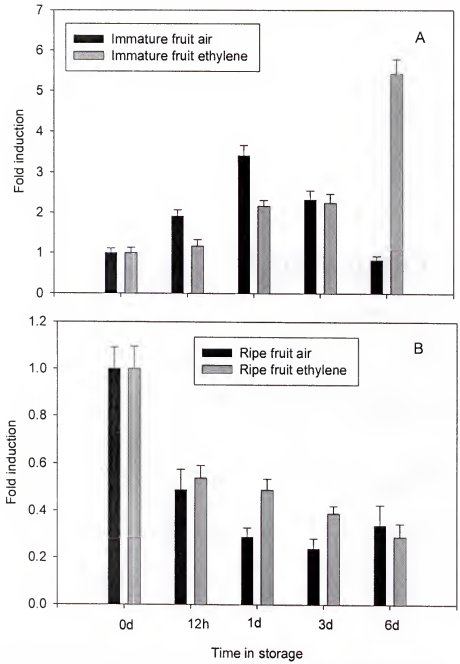

Figure 5-3. Northern blot analysis with a tomato β-gal probe (TBG4) of total RNA (10 μg) from air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3, and 6 days. Immature fruit (A), ripe fruit (B).

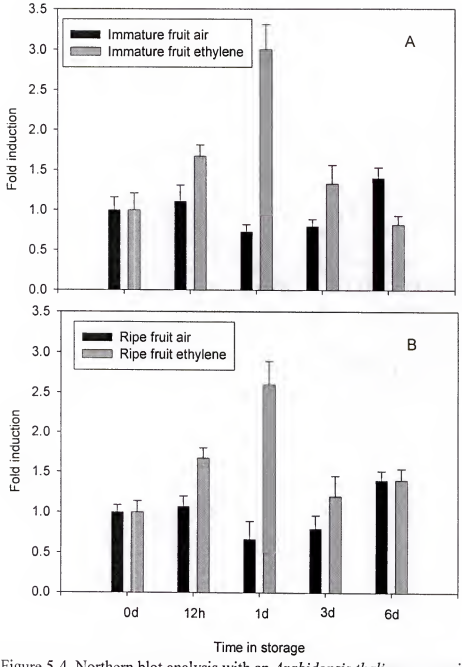

Figure 5-4. Northern blot analysis with an *Arabidopsis thaliana* expansin probe (*AtEXP1*) of total RNA (10 µg) from air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3, and 6 days. Immature fruit (A), ripe fruit (B).

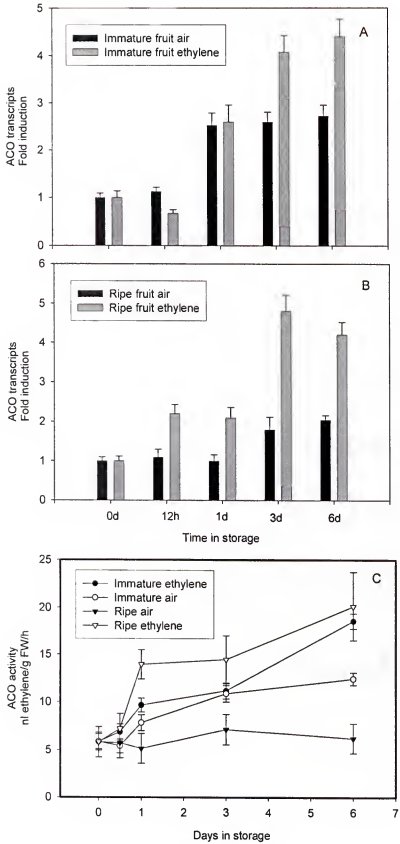
Immature fruit air
Immature fruit ethylene
A
ACO transcripts
Fold induction
Ripe fruit air
Ripe fruit ethylene
B
ACO transcripts
Fold induction
0d
12h
1d
3d
6d
Time in storage
Immature ethylene
Immature air
Ripe air
Ripe ethylene
C
ACO activity
nl ethylene/g FW/h
Days in storage

Figure 5-5. ACC oxidase transcript levels and enzyme activities in air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3 and 6 days at 20 ^{O}C. Northern analysis of immature fruit (A), ripe fruit (B), ACC oxidase activity in immature and ripe fruit (C).

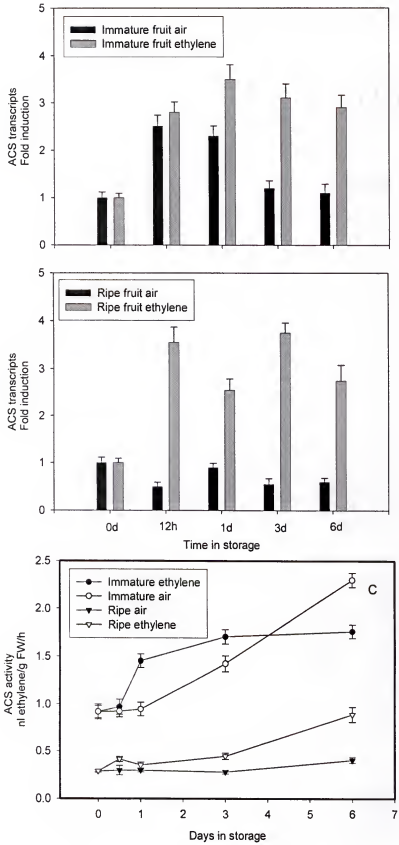
Immature fruit air
Immature fruit ethylene
ACS transcripts
Fold induction
Ripe fruit air
Ripe fruit ethylene
ACS transcripts
Fold induction
0d
12h
1d
3d
6d
Time in storage
Immature ethylene
Immature air
Ripe air
Ripe ethylene
C
ACS activity
nl ethylene/g FW/h
Days in storage

Figure 5-6. ACC synthase transcript levels and enzyme activities in air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3 and 6 days at 20 ^{O}C. Northern analysis of immature fruit (A), ripe fruit (B), ACC synthase activity in immature and ripe fruit (C).

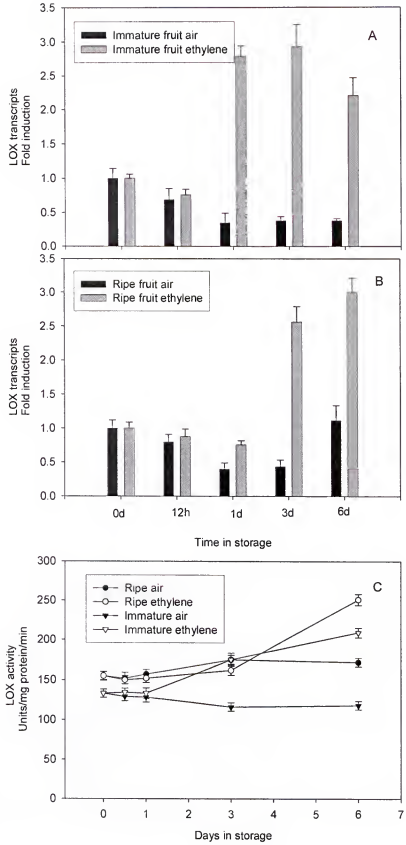
Immature fruit air
Immature fruit ethylene
A
LOX transcripts
Fold induction
Ripe fruit air
Ripe fruit ethylene
B
LOX transcripts
Fold induction
0d
12h
1d
3d
6d
Time in storage
Ripe air
Ripe ethylene
Immature air
Immature ethylene
C
LOX activity
Units/mg protein/min
Days in storage

Figure 5-7. Lipoxygenase transcript levels and enzyme activities in air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3 and 6 days at 20 °C. Northern analysis of immature fruit (A), ripe fruit (B), Lipoxygenase activity in immature and ripe fruit (C).

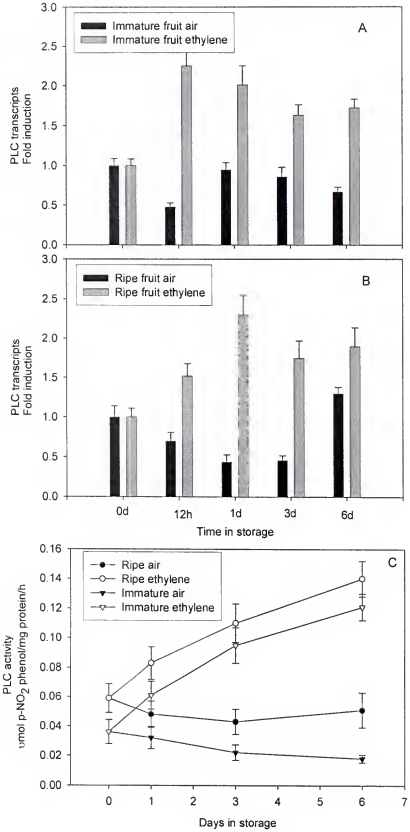
Immature fruit air
Immature fruit ethylene
A
PLC transcripts
Fold induction
Ripe fruit air
Ripe fruit ethylene
B
PLC transcripts
Fold induction
0d
12h
1d
3d
6d
Time in storage
Ripe air
Ripe ethylene
Immature air
Immature ethylene
C
PLC activity
umol p-NO2 phenol/mg protein/h
Days in storage

Figure 5-8. Phospholipase C transcript levels and enzyme activities in air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3 and 6 days at 20 °C. Northern analysis of immature fruit (A), ripe fruit (B), phospholipase C activity in immature and ripe fruit (C).

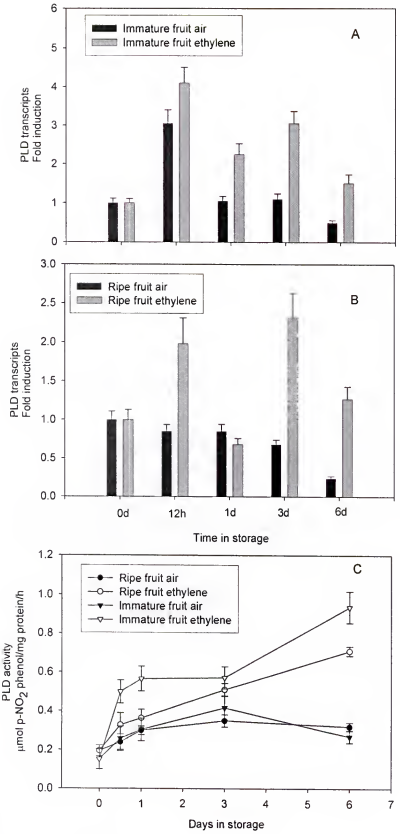
Immature fruit air
Immature fruit ethylene
A
PLD transcripts
Fold induction
Ripe fruit air
Ripe fruit ethylene
B
PLD transcripts
Fold induction
0d
12h
1d
3d
6d
Time in storage
Ripe fruit air
Ripe fruit ethylene
Immature fruit air
Immature fruit ethylene
C
PLD activity
µmol p-NO2 phenol/mg protein/h
Days in storage

Figure 5-9. Phospholipase D transcript levels and enzyme activities in air- and ethylene-treated watermelon fruit stored for 0, 12 hours and 1, 3 and 6 days at 20 °C. Northern analysis of immature fruit (A), ripe fruit (B), phospholipase D activity in immature and ripe fruit (C).

CHAPTER 6
CHARACTERIZATION OF WOUND-REGULATED CDNAS AND THEIR EXPRESSION IN FRESH-CUT PAPAYA FRUIT

Introduction

Fresh-cut fruits and vegetables are those that have been subjected to washing, sorting, trimming, peeling, slicing, coring etc. While fresh-cut commodities retain the quality and sensory attributes of the intact commodity, they show significant differences in physiological behavior. Refrigerated storage is required and employed extensively for quality maintenance and shelf-life of fresh-cut produce; however the chill sensitivity of fruits and vegetables of tropical origin necessitates new approaches to the maintenance of quality in these highly perishable commodities.

The physiological bases of deterioration and texture loss in fresh-cut fruits are unknown, but likely involve changes affecting both cell walls and membranes (Chapter 3, Picchioni et al. 1996, Cartaxo et al. 1997). In fresh-cut papaya, enhanced activities of polygalacturonase, α and β-galactosidase, lipoxygenase, phospholipases D and C in response to fresh-cut processing have been noted (Chapter 3). Plants respond to mechanical wounding with the induction of numerous genes. The first identified wound-inducible defense proteins in plants include proteinase inhibitors I and II from potato tubers and tomato leaves (Graham et al. 1986, Ryan 1990). In Arabidopsis, many genes are induced by mechanical wounding (reviewed in Reymond and Farmer 1998). The expression of many of these genes are induced by treatment with jasmonic acid (JA) or its precursor oxophytodienoic acid. These compounds, which are both members of the

jasmonate family (Creelman and Mullet 1997, Farmer et al. 1998), are essential in vivo regulators of defense gene expression (Reymond and Farmer 1998). Other signals and stimuli also lead to the expression of genes in wounded plant tissues, although the relative contribution of molecules such as ethylene (O'Donnell et al. 1996, Rojo et al. 1999) and abscisic acid (Pena-Cortes et al. 1989, Birkenmeier and Ryan 1998) and of electrical signals (Wildon et al. 1992) is still unclear. The importance of water stress/hydraulic pressure changes to gene expression during wounding has received even less attention (Malone and Alarcone 1995). It has been shown that wounding triggers an increase in the endogenous levels of the plant growth regulator jasmonic acid (JA) (Creelman et al. 1992, Albrecht et al. 1993, Laudert et al. 1996), and this increase is required for gene activation upon wounding (Pena-Cortes et al. 1993). Application of exogenous JA or its methyl ester at physiological concentrations can induce a variety of genes (Mason and Mullet 1990, Farmer et al. 1992). In potato tubers and tomato leaves, proteinase inhibitor genes can also be activated by oligosaccharides generated from both plant and pathogen cell walls by the action of polygalacturonases (Bishop et al. 1981) and by the 18-amino acid polypeptide systemin (Pearce et al. 1991).

The objective of this study is to characterize differentially expressed genes induced in response to fresh-cut processing (wounding) in papaya fruit by means of mRNA differential display (Liang and Pardee 1992). The identification of genes and gene products involved in the deterioration of fresh-cut tropical fruits might have significance for extending their shelf life, and provide information of more value to long-term goals of designing or selecting cultivars through genetic transformation with resistance or tolerance to deterioration.

Materials and Methods

Plant material and processing procedures

Papaya (*Carica Papaya*, var. Sunrise Solo) fruit originating from Belize were obtained from Brooks Tropicals, Homestead, Florida, USA. Fruit were sorted, washed with tap water, and rinsed with chlorinated water containing 150 μL L^{-1} free chlorine as were the cutting surfaces and cold room interior prior to cutting. The fruit were stored at 20 °C until they reached 60 to 70% yellow skin color. Fresh-cut processing was performed as described (Chapter 3). The tissue pieces were randomized and stored in vented plastic containers for 12 h, 1, 2, 4 and 8 days at 5 °C. Intact fruit stored under similar conditions served as controls. At the indicated intervals, fruit pieces were removed from storage, immediately frozen in liquid nitrogen, and analyzed as described below. At each sampling period, intact fruit were peeled, cut into pieces and immediately frozen.

Total RNA was isolated, quantified and its integrity was verified as described in Chapter 5. The total RNA was used for both Northern analysis and mRNA Differential Display as described below.

Differential Display of mRNA

Total RNA extracted from intact and fresh-cut fruit stored for 12 hours was used for differential display analysis. Fifty μL of total RNA (30 μg), 5.7 μL of 10X reaction buffer (GenHunter) and 10 units of DNase I were mixed and incubated at 37 °C for 30 minutes in order to eliminate genomic DNA contamination. After addition of 40 μL of phenol/chloroform (3:1, v/v) to remove DNase I, the samples were incubated on ice for 10 minutes and centrifuged at 4 °C for 5 minutes at max speed. The supernatant was collected and 5 μL of 3 M NaOAc and 200 μL ethanol were added and mixed. After

incubation at -80 °C for 1 hour, the samples were centrifuged for 10 minutes at 4 °C to pellet the RNA. The supernatant was removed, and the RNA pellet was rinsed with 0.5 ml of 70% ethanol. The RNA pellet was re-dissolved in 20 µL of diethylpyrocarbonate-treated water. The DNA-free RNA was re-quantified and RNA integrity was verified as described (Chapter 3).

Two µL (0.1 µg/µL DNA-free total RNA was reverse transcribed in a reaction mixture (9.4 µL water, 4 µL 5X RT buffer (125 mM Tris-Cl, pH 8.3, 188mM KCl, 7.5 mM $MgCl_2$, 25 mM DTT), 1.6 µL dNTP mix and 2 µL H-T_{11}M primer) of 20 µL including MMLV reverse transcriptase with subsets of specific 1-base anchored oligo (dT) primers (H-T11G, H-T11A, or H-T11C; GenHunter) that recognize different fractions of the total poly (A)+ RNA population. The reaction mixtures were incubated at 65 °C for 5 min, 37 °C for 60 min, 75 °C for 5 min followed by a 4 °C soak. The resulting cDNA was amplified with a combination of the flourescein-labeled anchored oligo(dT) primer and a 13-base pair (bp) primer of an arbitrary sequence (one of H-AP17-H-AP24, GenHunter) by a polymerase chain reaction (PCR) with Taq DNA polymerase (Qiagen). PCR was performed in a 20-µL reaction mixture comprised of 2 µL of cDNA solution, 0.2 µM anchored oligo(dT) primer, 0.2 µM arbitrary primer, 0.12 µM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin and 1 unit of Taq DNA polymerase. PCR conditions were as follows: 40 three step cycles of a 30-s denaturation at 94 °C, a 2-min annealing at 40 °C, and a 60-s elongation at 72 °C, and a final 5 min elongation at 72 °C in a thermal cycler (Techgene). Amplified cDNAs were separated on a 6% denaturing polyacrylamide DNA sequencing gel (4 mm) containing 7 M urea as described (Sambrook et al. 1989). Polyacrylamide gel

(acrylamide:bisacrylamide 19:1) was prepared in 1X TBE buffer (0.1 M Tris, 0.09 M Boric acid, 2 mM EDTA). Two μL of loading dye (99% formamide, 1mM EDTA, pH 8.0, 0.009% xylene cyanole, 0.009% bromophenol blue) and 3.5 μL of each of the PCR reactions were mixed and incubated at 80 °C for two minutes immediately before loading onto the sequencing gel. The samples were electrophoresed at 60 watts constant power until the xylene cyanol dye reached the bottom of the gel. The gel was scanned on a fluorescence Imager with 525 nm filter following manufacturer's instructions (FMBIO). Differentially expressed bands of interest were cut from the gel and amplified cDNA was eluted with 50 μL of dH_2O by boiling for 15 minutes. Re-amplification was carried out using eluted DNA as a template under the same PCR conditions and same primer combination except unlabeled H-T11A, H-T11C and H-T11G anchor primers were used instead of Fluorescein-labeled anchor primers.

Thirty μL of the PCR samples were run on a 1.5% agarose-TBE gel and stained with ethidium bromide. After confirmation of the size of re-amplified PCR products with their sizes on the original DNA sequencing gel, the reamplified cDNA probes were extracted from the agarose gel using QIAEX kit according to manufacturer's instructions (Qiagen). The extracted cDNA was labeled with Digoxygenin dUTP using DIG-High Prime labeling kit following manufacturer's instructions (Roche). The labeled probes were used to verify the differential gene expression and the change in gene expression through 8 days of storage by Northern Blot Analysis as described in Chapter 5 with the exception that 15-20 μg total RNA was used.

cDNA Cloning

After confirmation of differential gene expression, the re-amplified PCR products were cloned via either PCR-TRAP (GenHunter) or TA Cloning System (Invitrogen). In

the case of PCR-TRAP cloning system, ten μL dH_2O, 2 μL 10X ligase buffer (500 mM Tris-Cl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 500 μg/ml BSA), 5 μL PCR product and 1 μL T4 DNA ligase were added in order and mixed. The samples were incubated overnight at 16 °C and the GH competent cells (genotype: del (lac-pro) ara thi (ϕ80dlacZdelM15)) were transformed following manufacturer's instructions (GenHunter). The bacterial cells were grown for 16 hours at 37 °C with suitable aeration on LB-tet plates containing 20 μg/mL tetracycline, 1.5% bacto-agar, 1% bacto-trypton, 0.9% NaCl and 0.5% bacto-yeast extract. The plasmids containing the DNA insert were identified by the colony-PCR method using primers that flank the cloning site of the PCR-TRAP vector (GenHunter). All PCR products were analyzed on a 1.5% agarose gel with ethidium bromide staining and compared to the molecular weight marker.

After a plasmid was determined to contain an insert of interest, the corresponding colony was re-streaked to single colonies on a new LB-tet plate and incubated at 37 °C overnight. A single tet colony was inoculated into a 5 mL LB culture and used for plasmid miniprep using Wizard plus SV Miniprep kit following manufacturer's instructions (Promega). The plasmids were sequenced (ICBR, University of Florida) using Lseq and Rseq primers from Aidseq primer set C (GenHunter) with flourescent dideoxy terminator method of cycle sequencing on a Perkin Elmer, Applied Biosystems Division (PE/ABd) 373A or 377 automated DNA sequencer, following Abd protocols (Smith et al. 1986, McCombie et al. 1992). All consensus sequences were generated using the Sequencher Software from Gene Codes. The sequences were compared to the nucleotide and protein sequences available in the database using BLAST programs with default settings (Altschul et al. 1997).

Results

Fresh-cut fruits deteriorate more rapidly than their intact counterparts. A possible role of membrane deterioration through enhanced lipase activity detected in fresh-cut fruit as compared to intact fruit has been proposed (Chapter 3). To determine other genes possibly involved in rapid tissue deterioration of fresh-cut fruits, fresh-cut papaya was stored at 5 ^{o}C for 12 hours together with the intact fruit (control). The mRNAs from the fresh-cut and intact fruit were reverse-transcribed into their corresponding cDNAs, and then PCR amplification was carried out with three oligo d(T) and eight arbitrary primers in all possible combinations. Parallel comparisons of fresh-cut and intact (control) fruit differential display (DD-PCR) profiles revealed differences indicative of a wound-induced alteration of fresh-cut fruit RNA population. In total, approximately 2854 products were displayed, 36 of which were induced or upregulated and 32 depressed or downregulated. Twenty-two fragments were differentially displayed as specifically expressed cDNAs in the fresh-cut fruit but not in the intact fruit. The differentially expressed fragments of interest were isolated and were reamplified using the same primer combinations as in the first PCR and subsequently cloned into the plasmid vector. Northern blot analysis for RNA from intact and fresh-cut fruit was performed using these cloned fragments as probes. To determine the expression patterns of each of the isolated clones, RNA was extracted from intact and fresh-cut fruit stored for 1, 2, 4 and 8 days at 5 ^{o}C and used for Northern blot analysis. The cloned PCR products were sequenced and the nucleotide sequencing revealed that each of the cDNA clones contained at the 5' end the sequence of the arbitrary primer and at the 3' end a sequence complementary to the anchored primer utilized in the DDRT-PCR.

Table 6-1 summarizes the cDNAs isolated from the fresh-cut fruit stored for 12 hours at 5 °C by the differential display strategy. A total of 16 cDNA products of interest were excised, and 14 of these were successfully re-amplified and cloned. The majority of these represented DD-PCR products with specific accumulation in fresh-cut fruits (Figures 6-1, 6-2). Two represented a DD-PCR product with a repressed accumulation and five with an induced or enhanced accumulation. The insert of each of these clones was sequenced (Fig. 6-4). Each of the 14 cDNA sequences was submitted to the NCBI server for comparison to sequences residing in the GenBank databases using BLAST programs (Altschul et al. 1997).

The nucleotide sequence of the partial cDNA PC18-1 was similar to that of mitogen- and stress-activated protein kinase from *Rattus Norvegicus* and *Mus musculus*, respectively (Table 6-1). At the amino acid level, PC18-1 shared 33% identity and 58% similarity to a limonene cyclase-like protein in rice (*Oryza Sativa*). The nucleotide sequence of the cDNA insert PC17 showed significant similarity to the mRNA sequence of *Homo sapiens* A kinase (PRKA) anchor protein 2 (Table 6-1). However, PC17 did not show a significant homology to any known proteins in the database. The partial cDNA, PC23 shares 41% amino acid sequence identity with the carboxy-terminal portion of an unknown protein from *Homo sapiens* (Table 6-1). However, at the nucleotide level, it shared 88% identity with *Arabidopsis thaliana* putative CAAX prenyl protease. The PA17 insert shares 88, 87 and 89% identity at the nucleotide level to *Pisum sativum* lipoxygenase *LOXN2* mRNA, *Vicia faba* lipoxygenase mRNA, and *L. culinaris* lipoxygenase mRNA, respectively (Table 6-1). The predicted amino acid sequence of PA17 was also similar to that of lipoxygenases from various plant species.

Among the four sequences that were similar to papaya sequences, PC18-2 showed 95% sequence identity to a *Carica papaya* membrane channel protein that is a probable water channel protein (Table 6-1). The nucleotide sequence of PC18-1 was similar to that of plasma membrane channel protein genes from various plant species including *Carica papaya.* PC18-3 shares 86, 77 and 77% identity with superoxide dismutase from *Carica papaya*, *Cicer arietinum* and *Mesembryanthemum crystallinum,* respectively, at the amino acid level (Table 6-1). The nucleotide sequence of PC18-3 also showed significant similarity to that of various plant superoxide dismutases. At the nucleotide level, the partial cDNA PG23 showed 94% identity with mRNA sequence of *Carica papaya* 1-aminocyclopropene 1-carboxylic acid synthase 2 (*ACS2*)(Table 6-1). The amino acid sequence of PG23 was also similar to that of ACC synthases from various plant species including *Carica papaya*. The partial cDNA clone PG17 showed 86% identity to *Carica papaya* β-galactosidase at the amino acid level (Table 6-1). A high nucleotide sequence similarity to *Carica papaya* β-galactosidase mRNA sequence was also observed. The partial cDNA clone PA19-3 showed 93%, 84% and 82% identity at the nucleotide level to *Castanea sativa*, *Solanum tuberosum* and *Lycopersicon esculentum* calmodulin mRNA sequence (Table 6-1). Similarly, the amino acid sequence of PA19-3 shared high sequence homology with those of calmodulin proteins from various plant species. Although demonstrating low sequence similarity to *Homo sapiens* BAC clone RP11-618K19 at the nucleotide level, PC18-4 did not show significant homology to any known proteins in the database at the amino acid level (Table 6-1). No significant similarity to any sequence residing in the databases was found for the partial cDNAs PA19-1, PA22,

PC18-5 and PA19-2 (Table 6-1). It is possible that the short length of these differential display products prevented a higher rate of putative identification.

Northern blot analyses

The majority of the isolated DD-PCR cDNAs correspond to mRNAs either expressed only in fresh-cut fruit or whose abundance increased in fresh-cut fruit. Northern blot analyses were, therefore, carried-out to confirm that the corresponding genes were expressed in a fresh-cut (wounding) dependent manner. Of the 13 cDNAs, the expression of the genes corresponding to 5 of these was upregulated in fresh-cut fruit tissue (Figure 6-3). Two genes, PC17, PC18-5 and PA19-3 gave no signal on Northern blots. These may possibly represent genes expressed at a very low level in fresh-cut fruit and not readily detected by northern blot analyses.

Genes corresponding to PC18-1, PA19-1, PA17, PA19-2, PC18-2, and PC18-3 were specifically expressed in the fresh-cut fruit (Figure 6-3). PA22 was expressed in intact fruit after 8 days of storage, but induced in fresh-cut fruit within 12 hours, reaching a maximum level after 24 hours. Genes corresponding to PG17, PG23, PC23 and PC18-4 were expressed in both intact and fresh-cut fruit during storage but the levels in fresh-cut fruit were higher than the levels detected in intact fruit. PG17 was also detected in control fruit at Day 0 of storage. PA19-1 expressed at a low level only at Days 1 and 2 of storage in fresh-cut fruit. The low levels of expression observed for some of the cDNAs may result, in part, from the insensitivity of northern blot hybridization analyses with regard to detecting changes in RNA levels that can be readily demonstrated by PCR.

Discussion

Fresh-cut fruit deteriorate more rapidly than their intact counterpart (Chapter 3). The analysis of the changes in enzyme patterns in fresh-cut versus intact fruit during 8

days of storage (Chapter 3) revealed that the activity of α- and β- galactosidases, lipoxygenase, phospholipase D and polygalacturonase were enhanced in response to fresh-cut processing (wounding), suggesting an involvement of membrane and cell wall degradation in the rapid softening and deterioration of fresh-cut fruit as compared with intact fruit. In the present study, differential display was used to visually examine and compare the patterns of expressed mRNAs in fresh-cut papaya fruit with that of intact fruit. Subsequently, a number of fresh-cut responsive partial cDNAs were isolated and the expression of the corresponding genes was examined in fresh-cut and intact fruits. Fourteen partial genes responsive to fresh-cut processing have been characterized.

The cDNA clone PC18-2 that is expressed only in fresh-cut fruit, reaching a maximum after 4 days of storage, showed significant homology to *Carica papaya* probable membrane channel protein possibly functioning as a water channel (Agre et al. 1999). Aquaporins are central components in plant water relations and function as selective channels or relatively non-selective channels for the transfer through the bilayer water and other small non-electrolytes (Agre et al. 1999, Borgni and Agre 2001). They also allow ion permeation (Lee et al. 1995, Yasui et al. 1999). Aquaporins have been localized to cell membranes (Tyerman et al. 2002) and might be involved in compatible solute distribution, gas transfer (CO_2, NH_3) as well as in micronutrient uptake (boric acid).

The isolated putative calmodulin gene PA18-3 showed no transcript accumulation when determined using Northern blots, possibly due to low level of expression of the corresponding gene. Plant calmodulins show up to 22% divergent amino acid residues and seem to play different roles and have various target enzymes (Lee et al. 1995).

Calmodulin is a key factor in Ca^{2+} sensing and transport. The calmodulin-dependent regulation of protein kinases illustrates the potential mechanisms by which calmodulin can recognize and generate affinity and specificity for effectors in a Ca^{2+}-dependent manner (Knight 2000). The transcriptional upregulation of calmodulin genes through a wound stimulus or in response to treatment with systemin, methyl jasmonate or linoleic acid has been described for tomato leaves (Bergey and Ryan 1999). These authors have hypothesized that calmodulin gene expression may be associated with the signaling cascade that activates defense genes in response to wounding.

The partial cDNA clone PC17 showed no transcript accumulation on Northern analysis and was not significantly similar to any known proteins at the amino acid level. The clone did, however, show low homology at the nucleotide level to *Homo sapiens* A kinase (PRKA) anchor protein 2. These proteins are a group of structurally variable proteins with the common function of binding to the regulatory subunit of protein kinase A and targeting the holoenzyme to discrete locations within the cell (Dong et al. 1998).

A clone encoding proteins normally associated with plant defense has also been identified. The partial cDNA18-1, specifically expressed in fresh-cut fruit showing maximum transcript accumulation after 8 days of storage and demonstrated significant homology to mitogen- and stress-activated protein kinases from various species. Mitogen-activated protein kinase cascades are major downstream components of receptors or sensors that transduce extracellular stimuli into intracellular responses in yeast and animal cells and possibly have similar functions in plants since the basic assembly of MAPK cascades is conserved in all eucaryotes (Reviewed in Zhang and Klessig 2001). These proteins are activated by cellular stresses including wounding

(Romeis et al. 1999, Kovtun et al. 2000, Nuhse 2000). cDNA18-1 also showed significant homology to a limonene synthase like protein of *Oryza sativa.* Limonene synthase is a monoterpene cyclase, one of a subset of terpenoid cyclases that convert the allylic pyrophosphate to the respective mono, sesqui, and di terpene natural products in plants and microorganisms (Savage et al. 1994). Limonene synthase catalyzes the cyclization of geranyldiphosphate to yield the olefin 4(5)-limonene, an intermediate in the biosynthesis of the monoterpenoid perillaldehyde.

An increase in ethylene production in response to fresh-cut processing (wounding) has been reported in both fruit and vegetable tissues (Abeles et al. 1992 Rosen and Kader 1989) including papaya fruit (Paull et al. 1997), and it was anticipated that messages encoding ethylene biosynthetic enzymes would be among the differentially expressed genes in fresh cut papaya. The current study yielded a single clone, PG23, encoding the enzyme ACC synthase 2 that participates in the ethylene biosynthetic pathway by catalyzing the conversion of SAM to ACC (Adams and Yang 1979, Lelievre et al. 1997). Transcripts for the gene corresponding to PG23 appeared after 12 hours in fresh-cut and 24 hours in intact fruit, reaching maximum levels after 2 days of storage. The expression of the gene in intact fruit could be due to the binding of the probe to other ACC synthase isoform(s) or the induction of the gene due to low-temperature stress. Wound-inducible genes for ACC synthases have been cloned from many plant species including tomato (Lincoln et al. 1993), soybean (Liu et al. 1993) and winter squash (Watanabe et al. 2001). A significant increase in ACS activity in response to wounding was also observed in the present study, further supporting the wound induction of ACS (Chapter 3).

The gene corresponding to PG17 expressed in both fresh-cut and intact fruits, reaching maximum levels after 24 hours in fresh-cut and 48 hours in intact fruit. The levels of transcripts in fresh-cut tissue, however, were higher than those of intact fruit. An increase in β-gal activity in fresh-cut fruit during storage has been reported previously (Chapter 3). β-gal has been reported to act on cell wall beta (1-4) galactan polysaccharides in a number of fruits, including papaya (Ali et al. 1998), releasing monomeric galactose. β-galactosidase has also been proposed to contribute to membrane galactolipid modification through both hydrolytic and glycosyl transferase reactions (Onishi and Tanaka 1995, Bonnin et al. 1995, Yoon and Ajisaka 1996, Kajihara et al. 2000, Zeng et al. 2000).

The partial cDNA PA17 expressed at high levels in fresh-cut fruit, with the maximum expression noted after 24 hours of storage. This gene was similar to *LOXN2* of *Pisum sativum* that has been isolated from pea nodules (Wisniewski et al. 1999). Lipoxygenases (LOX) are non-haem containing dioxygenases that catalyze the peroxidation of unsaturated lipids (Feussner and Kuhn 2000). LOXs have been implicated in many physiological processes including wounding (Geerts et al. 1994, Bell et al. 1995, Royo et al. 1996). In response to physical wounding, LOX could be involved either positively, through its role in the production of defense-related signaling molecules (Creelman et al. 1992, Albrecht et al. 1993, Laudert et al. 1996), or negatively through participation in autocatalytic peroxidation reactions (Hildebrand 1989). LOX hydroperoxides can contribute to tissue damage through inactivation of protein synthesis and deterioration of cellular membranes. Consistent with the wound-induced LOX

mRNA accumulation, an increase in LOX activity has been observed in response to fresh-cut processing during storage of fresh-cut papaya fruit (Chapter 3).

The gene corresponding to PC18-3 expressed only in fresh-cut fruit with the maximum induction after 12 hours of storage and showed high homology to superoxide dismutases from various plant species. Superoxide dismutases (EC 1.15.1.1, SOD) dismutate oxygen radicals and thus prevent the lethal effect of oxygen radicals in aerobic organisms. SODs are metaloproteins and are classified into three types depending on the metal (Mn, Fe, Cu/Zn SOD) found at the active site (Fridovich 1986). SOD activity in plants increases in response to various environmental and chemical stimuli (Fridovich 1986, Perl-Treves and Galun 1991). An increase in SOD activity in response to wounding was reported in many plant species including pine needles (Karpinska et al. 2001), Arabidopsis plants (Abarca et al. 2001), cell cultures of cassava (Lee et al. 1999), and tomato leaves (Perl-Treves and Galun 1991).

The transcript levels of the gene corresponding to PC23 increased in fresh-cut fruit after 12 hours and in intact fruit after 2 days of storage and showed homology at the nucleotide level to a putative *Arabidopsis thaliana* CAAX prenyl protease. CAAX prenyl proteases have been suggested to remove the CAAX residues from proteins as a posttranslational modification for correct localization and full activity of mature proteins in yeast (Schmidt et al. 1998).

The biochemical function of the four clones designated as PA19-1, PA22, PC18-5 and PA19-2 are unknown. The rapid rate at which plant genes are being isolated, however, suggests that these sequences may soon be assigned a putative identity/function by database comparisons. Among these, no transcripts corresponding to PA19-2 and

PC18-5 were detected on Northern blots. However, the transcripts of the gene corresponding to PA19-1 were detected in fresh-cut fruit after 1 and 2 days of storage. The gene corresponding to PA22 expressed constitutively in fresh-cut fruit during storage but showed a slight transcript accumulation after 8 day of storage in intact fruit.

Identifying wound-related transcripts that are abundant in fresh-cut papaya fruit will enable a more in-depth understanding of the physiological events that occur during storage of fresh-cut fruits. It is now possible to use the isolated cDNA clones in experiments designed to investigate patterns of tissue-specific gene expression as a result of physiological and environmental changes. As has been suggested in Chapter 3, the changes in cell walls and membranes due to the induction of the expression of cell wall and membrane hydrolases likely play a significant role in rapid deterioration of fresh-cut papaya fruit. The induction of genes homologous to lipoxygenase, beta-galactosidase, mitogen- and stress-activated protein kinases, superoxide dismutases, calmodulin and membrane channel proteins further support the role of cell wall and membranes. The induction of superoxide dismutase and lipoxygenase-like proteins in response to fresh-cut processing further suggests a possible involvement of free radical-induced membrane disintegration in the rapid softening and deterioration of fresh-cut fruit as compared with intact fruit.

Table 6-1. Homology search result for papaya fresh-cut (wounding) regulated partial cDNAs.

cDNA	Insert Size (bp)	Homology search result
PC18-1	272	*Rattus norvegicus* mitogen activated protein kinase
PC17	229	*Homo sapiens* A kinase (PRKA) anchor protein 2
PC23	248	*Arabidopsis thaliana* putative CAAX prenyl protease
PA17	589	*Pisum sativum* lipoxygenase
PA19-3	472	*Castanea sativa* calmodulin
PC18-2	446	*Carica papaya* membrane channel protein
PC18-3	541	*Carica papaya* superoxide dismutase
PG23	777	*Carica papaya* ACC synthase 2
PC18-4	245	*Homo sapiens* BAC clone GSI-195F7
PG17	409	*Carica papaya* beta-galactosidase
PA19-1	211	No homology
PA22	180	No homology
PC18-4	154	No homology
PA19-2	184	No homology

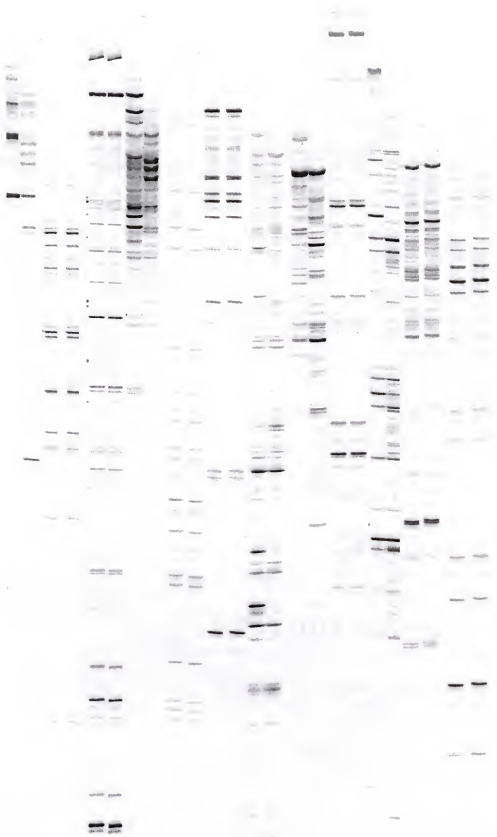

Figure 6-1. Differential display of RT-PCR products for intact and fresh cut papaya fruit stored at 5 ^{O}C for 12 hours.

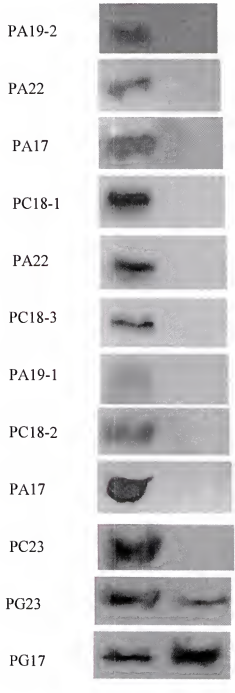

Figure 6-2. Northern blot analysis of gene expression on total RNA isolated from intact and fresh-cut papaya fruit stored for 12 hours.

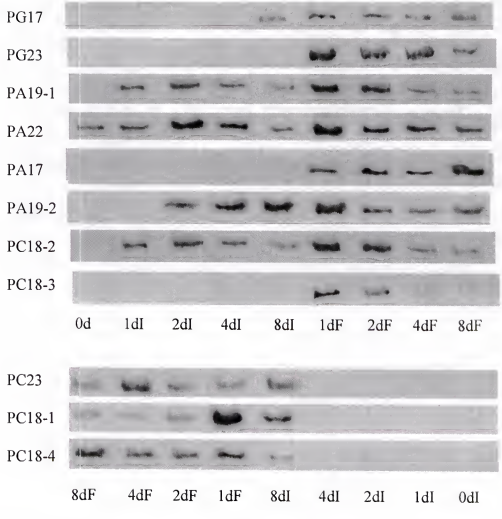

Figure 6-3. Expression of genes corresponding to DD-PCR products in intact (I) and fresh-cut (F) papaya fruit stored for 0, 1, 2, 4, and 8 days at 5 ^{o}C.

Figure 6-4. Nucleotide sequences of DD-PCR products.

PC18-1

TTCGAATCTCCGTAGTTCCAAAGGAGACAGCTCTGTGAAGAAGACCTCCGGG
TGGTTTGGGGGGTATCCTAAGGAGGCTGTCTGGGAGCTTCGCAGACACCTTG
GCTCTAAATTCTCCGGAAGAGGAATCCTGGTTGGCTCTCGACTGAATGGTGCT
TTTATCCCAAGTCATCCACCTGGAAAGGCTGTGTAAGCTTAGAGGCAACAAT
ATTGTGGAGAAGTTGTTCTTACTGCCAGTTGCATTATTAATCATCTGCCTCTA
AGGTAACATCTAAGCTTTTTTTTT

PG23

AAGCCAACATCTTCGAACCGATACTGTCAAAGGTTATGTTGGTCATCTTCGTC
AACGAGACAAGACCATCCACTTGGTCTGCGACGAAATCTATGCCGCCACCGT
CTTCAGCTCCCCGGAGTTCGTCAGCATCGCGGAGATCATCCAAGAAATTGAC
GTTAACACGACGACCTTATCCACATCCTCTACAGCTTGTCCAAAGATATGGGT
CATTCCCCTGGTTTCCGGGTAGGCATTGTGTATTCCTACAACGACGTCGTATT
AAGCTGTCGGCCGGCGGATGTCGAGCTTTGGGTTGGTCCTCGTCACAGACTC
AATATTTTCCTGAGCGACACTGCTGTCCGACGACGACGTTCGTCGATTACTTC
CTCTCGGGATAAGCTAACGAAGAGGCTGGCGAGCAAGACACCATAAACTCA
CCATGAGGGCTGGAGCAAGTGGGGATAAAGTCGCTTGAAAAGCAATGCCGG
ACTTTTATTGTGAATGGATGAGACCTGCGGCAGGCTCCTGGAAGATCCAACG
TCTGTTTGATGCAGAAATGAAGCTGTGGCCGGACCATCGTCAACGACGTGAA
ATGCTGAACGTGTCGCCGGGATCTTCCGTTCCACGTGGCGGAGCCGGAAAGG
TTGGTTTAGGGTTTGTTATTGCAACACATGGACGAATTCGTGCATGCATAGTC
ATGCATGCATGCAGCTTAAGCTCCATGCATGCACATGCATGCATGCATGTCAT
GCATATGCGCTTTTTTGCACTAGCCAAGCTTAAGCGTTTTTTTTT

PA17

AAGCTTACCAGGTCGATCAACTAAAAGCAAAAGATTCATGCCTGAACAAGGA
TCTCCAGAATAACGAAGAGTTCGCTAAGGACTACGAGAAGGCATATTTGAGT
ACAAACCACACCAATGAATGAAAGTCTTTGGAACTTGACAGTTAGGGAGGTA
TTCGTCAACACATGGTTCCGATGAGTAATACCTCGCACACAGAAATCGAACC
CGGTCGATCTTTGGACTTCGGACTCACACCGAATAGAGGCGTACAAAGAGTT
TGTCTGCGAACAAAGTCGGCAGTTGAAATTGAGAAAAAGCTCATTGAAGGGA
AGGAAAAACAACTGATGAGACATTGAGTAACCGATATGTGCCTGTGACGAAT
GCCATACACTCATACTTTATCCTTCCAGTGAAGCAGGATTGACTTTTAGAAGG
CATTCCCAACCAGTATCTCTATCTAAAATATGTAAGGTTGTTGTATTGTTGCT
ATGAATACATCGATGAGAACGAATCATATATTCCCCCATCCCCCATGTATGTA
TGTTACTAATGTATCCTTGTTTGATCACTGTAAATCAAAAACTACTATGTATG
TTCGTAAAAAAAAAAA

PC18-3

AAGCTTAGAGGCATTCAATCCTGCTGGCAAAGAACATGGTGCTCCAGAGGAT
GACATCCGTCATGCTGGTGACCTTGGAAATGTAAATGTTGGTGATGATGGCA
AAGTTAGCTTCTCAATTATCGACAGTCAGATTCCTCTTACTGGACCAAACTCC
ATTGTTGGAAGGGCTGTTGTTGTCCACGCTGATCCTGATGATCTTGGCAAAGG
GGGGCATGAGCTCAGTAAGACTACTGGAAATGCTGGGGGCAGAGTTGCTTGT
GGAGTCATCGGTCTCCAAGGTTGAAGAATCATTTCCAGAAATATTAGAATCA
GTTGAAAAGCCCGCGGATCACATGCTGCCTTACCGGTTTTTGGCAGTAAACTC
GGTGACTAGAAACTCTTTGAGTAAATGGAGATTACTTGTTTGTGTATTTTGGG
TTAGACTGGTTTTTGTGGTCTTAAAATTGTGAGTTGTTCTTGTTATTGCTCTGA
AATAGCTCTCCTCTTATCAGCATTATATCATCATCCCCTGAAATTTTCATGCCC
CGAAAAAAAAAAA

PC18-2

AAGCTTAGAGGCATTGGGATGACCATTGGATTTTCTGGGTAGGACCATTTATT
GGAGCAGCACTTGCGGCACTATATCAACAAGTTGTGATCAGAGCAATTCCTT
TCAAGTCCAAGTGCAAATTCCCAAGGTAATGGGTCTCCATAAAATATGTCATT
TAAGAATTTAAGATGGTGAAAAATGTAATATCATCAAATTTGAGCCCAGCCT
CTTCTTATTTCGCACGAACCTTGGGTTTCCTACTGGCTGTTTCTATTCTTCTAT
GGTCAATTATGTGTATTTTGTTGTTCTAAGAAATGTAAAAAATGGAAGTTTAG
TTCCTTTATCTTTGGGTTTGAAAAGAAGTTGGCTGAAAATGGTGGGGGCCATT
ATTGTGTTTGTATGGCTGTGATTTGTGCATGTCATTCTCAAATATAATAATAC
GCTAATTTCATGAAAAAAAAAAA

PC23

AAGCTTGGCTATGCTGCTCAA GTCCGGTTAA CTCGACTCTT GTACGGGTTG
ATGTCTTTTA GGTGACAGGT CGAAACAAAA CAAAAAAAAG TGTAACACCA
TATAGGCAGA GGCCTCATTT GTTGGTGTAT TACCTTATGC ATGCATGCAT
GCATGCATGC ATGCATGCAG CTTAAGCTCA TGCATGCACA TGCATGCATG
ATCGATATGC GCTTTTTTGC ATAGCCAAGC TTAGGGAAAA
AAAAAAAA

PC17

AAGCTTACCAGGTCCAGGCACGCCGGAAGCGAGAGGGCCCAGATTCGGACG
TTCACTTTCCTCCAGAGCTGATATACGCACGTTGCTTCGCGTATTCCGGACAT
TGCAGCGTAAGTTCGATGGGAACCTGACATGGATACGTATTGGGAACCATTA
CTGGGAGTCTTGAGTTAGCGAGCGGTATTATATGGCGCAATATCATGCTTACC
ACACGGTTGAAAAAAAAAAA

PC18-4

AAGCTTGGCTATGTCGCAGCATTATGCAATTTGCATGCAGGGCATGAAGACG
GTGCAGGGGCAGGGCCAAATGAGACGTAGCTGGTCGTCTCTCTGAACGGCAT
ACGGCTGGCATGGCTATGCAGAGGTTAAGACGTCCGCGCATAGCAATGATCC
GCATGCCTTGACATGGTTATCCGGAATATGCATTGGATGCATAAGGTCCGCA
ACTAACGTGCATGACATGGCATCCCGAAAAAAAAAAA

PC18-5

TTCGAATCTCCGTACAATATTGTGGAGAAGTTGTTCTTACTGCCAGTTTGCTC
CCATTATTTAAATATCATCATCCCGCGAGACGGGACCTATATATGCATAAATT
CGCAATCATCTGCCTCTAAAGCTTACCCTCTAAATCTTTTTTTTTTTT

PA19-1

AAGCTTATCGCTCGTTTTTACGAGAAAGCCGCTACATACAGATTAGATTAACC
CATTGTTTATATAACGACACGCCTAGAGACGACCGGGGCCAGTAGTAAATAA
CGAACGGAGAGGTGGTGCAGCACCAAGGACCCAAGATCTTTAAGGGACGAG
CCCCTTACGTTTAAGGCATGCATAAAAAAGCTTATCGCTCTTTAAAAAAAAA
AAA

PA22

TTCGAAAACTAGGCGCGAAGCTTTTGATCCCATGGAGAATCAGAGGCGGGAT
ATTGCATGCATGCATGCTATTAATTTATACAGGACAGAACTGCATAGCATGCC
ATGGGGATATTTGCATGGCATGCAGAGAAATGCCCGGGATGCATGCAATGCA
TGCCATGCATGATTTTTTTTTTT

PA19-2

AAGCTTATCGCTCTTGATTTATACGTCAGGACATAGACTACAGGCCCACAGTA
CATTGTACATTTACCAATATACCTCGAGAGGGCACCATTTCAGCATGAGGGC
ATATTGGAGAGCCCATAAAAAAGCTTATCGCTCTACGTACGTACGTATCTCGC
TCGCTATTCGTTTATAAAAAAAAAA

PG17

AAGCTTACCAGGTCTCGGAAGTAATCAAGAGGCTCATGTATTCAAATCAGAG
TCGGATTGCGCTGCATTCCTCGCAAATTATGACGCAAAATACTCTGTTAAAGT
GAGCTTTGGAGGCGGGCAGTATGACCTGCCGCCATGGTCCATCAGCATTCTTC
CGGACTGCAAAACCGAAGTTTACAACACTGCAAAGGTTGGTTCGCAAAGCTC
GCAAGTTCAGATGACACCAGTACATAGTGGATTTCCTTGGCAGTCATTCATCG
AAGAAACCACGTCTTCTGATGAGACCGATACAACTACATTGGACGGGTTGTA
TGAGCAAATAAATATCACTAGGGATACTACAGACTACCTTTGGTACATGACA
TGCATGCATGCATGCATGCATGCATGCATGCAAAAAAAAAAA

PA19-3

AAGCTTACCAGGTAGAGTTGGGAACTATCATGAGATCTTTGGGACAGAATCC
GAAGCTGAACTACACCACTTGATGAATGAAGTTGATGCTGATCAAGATGCGC
ACTATTGATATATCTGAGTTTCTGAATTTGATGCCACGAAAAATGAAGGATAC
TGATTCTGAGGAGGAACTCAAAGAGGGTTTAAATGTGTTAGATAAGGACCAG
AATGGCTTTATTTCTGCTGCAGAGCTTCGACTTGTAATGACAAATCTTGGGGA
AAGGCTGACAGATGAAGAAGTCGATGAGATGATACAGAAAGCAGATTTGGA
TGGTGATGGTCAGGTGAATTATCAGGAGTTTGTAGGAGCAATGTGCACCATG
CAAATCTCTACTGTGCTTTTCGTTTTGCTGATATCTGATGTAAAGGGACTGTC
AGATTATCCAGGAGTATCATTGTTTTCATAGTGTTTTTGGATTTGTAAAAAAA
AAAAA

CHAPTER 7
CONCLUSIONS

Fresh-Cut (Wound) Induction of Gene Expression and Enzyme Activities in Fresh-Cut and Intact Papaya Fruit

Fresh-cut papaya fruit demonstrated accelerated softening and tissue disintegration in response to fresh-cut processing during 8 days storage period as compared to intact fruit. Fruit firmness decreased nearly 36% after only 2 d and continued a steady decline throughout storage at 5 °C in fresh-cut fruit. However, tissue derived from intact fruit stored under identical conditions showed little change in firmness declining 19% by the end of the 8-day storage period.

Analysis of the cell-wall polyuronides revealed that total polyuronide content did not change in tissue of intact papaya stored for up to 8 d whereas a decline (10%) was noted for the fresh-cut fruit. In intact fruit, levels of chelator-soluble polyuronides did not change, whereas water-soluble polyuronides showed a significant increase after 8 d. In fresh-cut fruit, the levels of both water- and CDTA-soluble polyuronides increased significantly within the first 24 h and continued to increase with storage. Chelator- and water-soluble polyuronides from intact fruit showed little change in mol mass during storage. Mol mass downshifts in polyuronides in fresh-cut tissue were evident at 4 d of storage, with more extensive downshifts evident after 8 d suggesting enhanced depolymerization or solubility of inherently smaller polymers. Consistent with the participation of depolymerization was the significantly greater levels of PG activity in fresh-cut compared with intact fruit and the decrease in total uronic acids. The higher

levels of PG activity detected in fresh-cut fruit might represent a response to increased ethylene production due to the induction of ACC synthase (ACS) and ACC oxidase (ACO) activities. ACS and ACO activities increased markedly in fresh-cut and intact papaya fruit during storage and in response to fresh-cut. After 2 d of storage, levels of ACS activity in fresh-cut and intact fruit were 250% and 63% higher, respectively, compared with those of fruit prior to storage (Day 0). Although showing different patterns in intact and fresh-cut fruit, ACO activity increases were not correlated with the fresh-cut.

Other enzymes involved in pectin metabolism and depolymerization, including galactosidases and pectinmethylesterase (PME), were also examined. PME was not different in fresh-cut compared with intact fruit, with activity increasing through 2 d of storage in both tissues, and thereafter remaining constant suggesting that levels of extractable PME do not correlate with the softening and deterioration of fresh-cut compared with intact papaya fruit. Alpha- (α-Gal) and beta-galactosidase (β-Gal) activities were enhanced in fresh-cut compared with intact papaya. Over the entire storage period, α- and β-gal activities increased 147% and 116%, respectively, in fresh-cut fruit compared with 18% and 64% in intact fruit showing activity trends parallel to the firmness declines in the fresh-cut and intact papaya.

The change in the activities of lipases seem to be prominent in the rapid softening and deterioration of fresh-cut fruit relative to intact fruit. Lipoxygenase (LOX) activity accumulated dramatically in response to fresh-cutting, increasing more than 3 fold in the initial 24 h of storage. In contrast, LOX activity increased slightly in intact fruit during storage. PLC activity increased markedly (32%) in fresh-cut fruit within 24 h thereafter

decreasing towards the end of the storage. PLD activity in fresh-cut tissue increased 48% within 24 h and then remained relatively constant during the rest of the storage period. Although increased significantly within 4 d, PLD activity in intact fruit remained significantly lower than that of fresh-cut fruit throughout the storage.

Differential display analysis on fruit stored for 12 hours after fresh-cut processing revealed that 98 genes were differentially expressed in response to fresh-cut, of these 36 were upregulated and 32 were downregulated. 22 genes were specifically expressed in fresh-cut fruit but not in intact fruit. Among 98 differentially expressed genes, 16 cDNAs of interest were selected for further analysis and 14 of those were successfully cloned and characterized. The sizes of cDNAs ranged from 154 bp to 777 bp. The partial cDNAs showed significant homologies to signaling pathway genes, membrane proteins, cell-wall enzymes, proteases, ethylene biosynthetic enzymes, enzymes involved in plant defense responses and proteins with no known functions. Among characterized clones, PC18-1, PA17 and PA19-3 showed significant homology to *Rattus norvegicus* mitogen and stress activated protein kinases, *Pisum sativum* lipoxygenase, and *Castanea sativa* calmodulin respectively. The partial cDNA PC18-2 shared significant similarity to *Carica papaya* membrane channel protein. PG17 was similar to *Carica papaya* β-galactosidase. PC23 and PG23 showed significant similarity to *Arabidopsis thaliana* putative CAAX prenyl protease and *Carica papaya* ACC synthase 2 respectively. The partial cDNA clone PC18-3 was significantly similar to *Carica papaya* superoxide dismutase. The two partial cDNAs PC17 and PC18-4 demonstrated low sequence similarity to *Homo sapiens* A kinase (PRKA) anchor protein 2 and *Homo sapiens* BAC clone GSI-195F7 respectively. Four partial cDNAs PA19-1, PA22, PC18-5 and PA19-2 showed no homology to any

known sequences residing in GenBank databases. Northern blot analysis with the probe of each of the partial clone revealed that most of the genes corresponding to partial cDNAs expressed in a fresh-cut dependent manner. The genes corresponding to cDNAs that were up- or down-regulated in response to fresh-cutting (wounding) showed transcript accumulation in both intact and fresh-cut fruit. The transcripts for PC19-2, PC18-5, PA19-3 and PC17 were not detectable on northern blots and possibly these encode genes expressed at very low levels that are not readily detectable with northern blot analysis. The generally higher and more rapid accumulation of enzyme activities in fresh-cut tissue might represent an overall response to wounding. The rapidity of these increases, typically observed within 24 h of tissue wounding, argues that microbial proliferation, which becomes more problematic during long-term storage of fresh-cut fruit did not contribute to the enhanced activities of cell wall and membrane hydrolases. Moreover the variations observed between intact and fresh-cut fruit, stored under similar conditions, support the view that the firmness and enzyme trends in fresh-cut papaya are not a direct result of low-temperature stress. The rapid softening and deterioration of fresh-cut papaya possibly include membrane and cell wall catabolism accelerated or otherwise altered as a result of physical wounding. Additional evidence supporting the roll of cell wall and membranes in the rapid softening and deterioration of fresh-cut fruit was the induction of genes homolog to lipoxygenase, β-galactosidase, mitogen and stress activated protein kinases, superoxide dismutases, calmodulin and membrane channel proteins. The induction of superoxide dismutase and lipoxygenase-like proteins in response to fresh-cut processing further suggests a possible involvement of free radicals in the rapid softening and deterioration of fresh-cut fruit as compared to intact fruit.

Collectively, the data suggest that enhanced hydrolase activity and accelerated senescence are involved in the rapid softening and deterioration of fresh-cut papaya fruit.

Ethylene Induced Gene Expression and Enzyme Activities in Watermelon Fruit

Watermelon fruit displayed marked tissue softening and water-soaking in response to ethylene. Placental tissue water-soaking was visible following 3 days of ethylene exposure and acute tissue liquefaction had occurred by 6 days. The similar softening and deterioration were also observed for rind (pericarp) tissue.

The ethylene-induced decline in placental tissue firmness was correlated with changes in the activity of several cell wall enzymes; however, none of the enzymes demonstrated accumulation patterns in fruit of both maturity stages (immature versus ripe) that would suggest a direct role in placental tissue water-soaking. Ethylene-induced accumulation of α-gal and PME was limited to immature fruit. β-gal activity showed no change in either air- or ethylene-treated ripe fruit. The only consistent response among the cell wall enzymes to ethylene treatment in both immature and ripe fruit was increased PG activity. PG activity increased significantly in both immature and ripe fruits and in both air- and ethylene-treated fruits after 3 days of storage. Activity levels were significantly higher in ethylene- compared with air-treated fruit at Days 3 and 6 for immature and Day 6 for ripe fruit.

Northern blot analysis on ripe and immature watermelon fruit revealed that PG and EXP mRNA levels increased in response to ethylene exposure in both ripe and immature watermelon fruit suggesting a possible involvement of these two cell-wall enzymes in the watersoaking phenomena. Consistent with the enzyme activity data, the accumulation of β-gal and PME transcripts, were limited to immature and ripe fruit respectively. The lack of an induction of β-gal in ripe fruit and PME in immature fruit in response to ethylene

suggest that β -gal and PME possibly are not influential in ethylene-induced watersoaking in watermelon fruit. The changes in transcript levels and enzyme activities (PG, PME, α- and β-gal, EXP) induced by ethylene exposure were typically paralleled by similar though less marked changes occurring in air-treated fruit.

The softening of placental tissue was temporally correlated with significant mol mass downshifts in water- and CDTA-soluble polyuronides. The downshifts in mol mass were extensive, particularly in ripe fruit, with both water- and CDTA-soluble polymers fractionating near the inclusion limit of the column. In immature fruit, both CDTA- and water-soluble polyuronides increased significantly with time of storage in both air- and ethylene-treated fruit. Although ethylene effects were evident for both pectic fractions, the influence of ethylene compared with air on immature fruit was more dramatic for the CDTA-soluble fraction, showing 44% and 42% higher levels at Days 3 and 6, respectively. Water-soluble UA content did not change significantly in ripe fruit maintained in air. Ethylene-induced increases in water-soluble polyuronides were evident by Day 6, at which time levels were 58% higher than those from air-treated fruit. Total uronic acid (UA) levels were significantly higher in immature compared with ripe fruit. In air-treated immature fruit, total UA levels increased slightly (5%) during storage whereas ethylene-treated fruit showed a significant decline (10%). A decline in total UA was noted for both ethylene- and air-treated ripe fruit, though the decline was significantly greater for the ethylene-treated.

Analysis of the changes in the transcript and activity levels of ethylene biosynthetic enzymes revealed that ACO and ACS transcripts induced significantly in both ripe and immature fruit in response to ethylene exposure and was accompanied by enhanced

activity of both ethylene biosynthetic enzymes possibly resulting in elevated ethylene biosynthesis. Although a 2-5 fold increase in transcript levels were observed for both ethylene biosynthetic enzymes, the increase in their activities was approximately 5-10 fold suggesting an enhancement of the activity together with increased protein synthesis.

The analysis of enzymes targeting membrane lipids revealed significant inductions in the levels of both activity and transcripts of lipoxygenase (LOX), phospholipase C (PLC) and phospholipase D (PLD). Although remained relatively constant or decreased in air-treated fruit of ripe and immature watermelon fruit respectively, LOX mRNAs accumulated significantly in response to exogenous ethylene during storage indicating a possible involvement of LOX-dependent lipid hydrolysis to the water-soaking syndrome. Similar to transcript levels, LOX activity increased markedly during storage in response to ethylene in both ripe and immature watermelon fruit coinciding with the increase in transcript level thus further supporting the role of LOXs in water-soaking disorder in watermelon fruit. PLD and PLC transcript accumulation were an early response to ethylene, the transcript levels of which increased two and four fold respectively in immature fruit within 12 h accompanied by an increase in the activities of both lipases. Similarly, a significant but delayed increase in mRNAs and activities of both lipases were observed in ripe fruit indicating a possible membrane lipid degradation in response to exogenous ethylene.

The absence of significant increases in both mRNA and activities of LOX, PLD and PLC enzymes in air-treated fruit suggests that these lipases are induced by exogenously applied ethylene possibly resulting in membrane lipid hydrolysis and leading to the leakage of the cytoplasmic fluid into the apoplast, thus generating

watersoaking phenomena and the resulting change in apoplastic conditions may enhance cell wall hydrolase activity leading to extensive cell wall polysaccharide degradation.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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